Ecotoxicological analysis of cyanotoxins and antibiotics as a threat to environmental health and exploration of statistical methods for the analysis

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CONTENTS

ABSTRACT

Environmental pollution is linked mainly to human activities as well as natural processes, therefore it is an inevitable problem that is causing a massive burden on biodiversity, ecosystems, and human health. To track and investigate contaminants that are invading the aquatic environment, "ecotoxicology" or "toxicity assessment" is a useful tool for such aims. However, ecotoxicological studies require interdisciplinary knowledge, such as ecology, toxicity, biochemistry, statistic, and so forth, thus many gaps can be mined and addressed which we can depart from fundamental studies. Considering our laboratory conditions, we first perform the ecotoxicological research to identify the detrimental abilities of emerged contaminants cyanotoxins, and emerging contaminants antibiotics to sensitive organisms zooplanktonic species which play important roles in freshwater trophic levels. Besides, to exploit efficiently obtained data as well as to encourage the essential improvements of ecotoxicological data analysis, we apply powerful and rigorous statistical methods to interpret analyzed outputs. First, I provide general information and the importance of my academic field and its connection with environmental pollution. Besides, I also declare the motivation of Ph.D. research. Second, I provide intensive reviews with a huge meta-analysis of targeted contaminants antibiotics, and cyanotoxins, such as effect mechanisms, environmental pathways, and occurrences in aquatic environments. Third, the subchronic effects of the cyanobacterial crude extracts from microcystin-producing and microcystin-free cyanobacteria, with different microcystin concentrations (1, 10, and 50 μg L^{-1}) on *Daphnia magna*. The life-history trait responses of *D. magna* to cyanobacterial crude extracts were determined based on survival, reproduction, and somatic growth. In addition, the physiological response represented by the feeding rate of *D. magna* on green algae (*Scenedesmus* sp.) after exposure to both types of crude extracts was also estimated. Fourth, in the ecotoxicological context of 48-hour acute exposures, we investigate *Simocephalus vetulus* responses of mortality and thoracic limb rate to four fluoroquinolone antibiotics, tetracycline, and the antibiotic cocktail of those antibiotics at the concentrations of 0, 0.625, 1.25, 2.5, 5, 10, 20, and 40 mg L^{-1}. Fifth, the chronic toxicity test for *D. magna* was performed during 42 days under exposure to fluoroquinolone antibiotic ciprofloxacin and ofloxacin concentrations of 50, 500, and 5000 μg L^{-1}. The assessment endpoints were survival, maturity, fertility, and offspring degradations. Briefly, our studies in the PhD dissertation contribute the first and interesting results to the understanding of the toxicity of cyanotoxins and high-consumed antibiotics to sensitive organisms in aquatic environments. Besides, I am also successful in suggesting the application of rigorous statistics based on Frequentist methods for ecotoxicology.

Keywords: fluoroquinolones, cyanotoxins, life-history traits, physiology, ecotoxicology

1. INTRODUCTION

1.1. General backgrounds and objectives

1.1.1. The importance of ecotoxicology

The awareness of contaminants that induced the suppression of the ecosystem may be raised by Rachel Carson in the book "Silent Spring" (Carson, 2002). From this event, the scholars paid more attention to environmental chemicals in the environmental compartments and this is the root of the "ecotoxicology" term (Truhaut, 1977). Ecotoxicology, the subject of study of ecology and toxicology combinations, refers to the potential for biological, chemical, or physical stressors to affect ecosystems (Chapman, 2002). Therefore, the release of ecotoxicology is to deal with the big questions of whether and how xenobiotics cause risks for various ecosystems (Chapman, 2002).

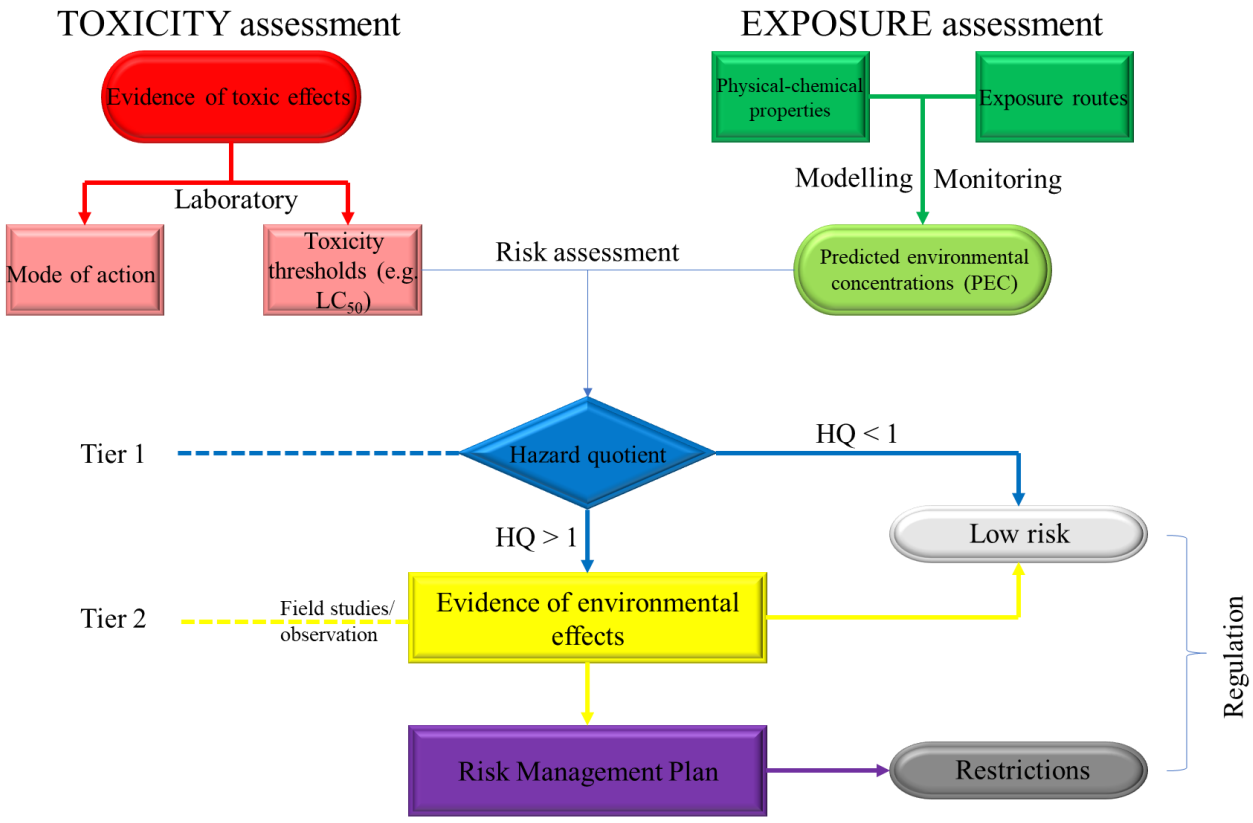

Fig. 1-1. Ecological risk assessment framework adjusted according to Sánchez-Bayo and Tennekes (2015). HQ = Hazard Quotient.

As shown in Fig. 1-1, ecotoxicology (or toxicity assessment) is one of two components of ecological risk assessment. It needs to be gently introduced that ecological risk assessment is a comprehensive work to evaluate the potential risks of specific activities of humans or nature which appear to cause negative effects on species, communities, or ecosystems (Graham et al., 1991). Therefore, ecological risk assessments are the big and important works of governments of almost all countries which must require interdisciplinary cooperation (U.S. EPA, 1998). Besides, ecotoxicological studies are performed in both the laboratory and field conditions (Storck et al., 2018) (Fig.1-2).

Fig. 1-2. Ecotoxicological studies in the laboratory (left) and field (right). Source: University of Exeter, UK, and IBACON, Germany.

Our research, herein, is to contribute the important, new, and interesting experiments based on the fundamental methodology which is favorable for our laboratory conditions, to investigate the potential risks of contaminants to sensitive organisms. We also believe that our results will support the evidence of contaminant effects, i.e. antibiotic and cyanotoxins, to decision-making in governments for ecological protections.

1.1.2. Antibiotics and cyanotoxin as emerging and emerged pollutants

Considering our laboratory conditions, in this research, we focus on two well-known contaminants in many environmental compartments, namely, antibiotic and cyanotoxin in which we pay more attention to antibiotics because they are the hot topic with many gaps that need to be disentangled.

The release of antibiotics is firstly to treat bacterial infections in humans that may be started to search since 1904 (Aminov, 2010). After evidence of efficacy and safety, antibiotics have been used in many fields related to the human production process, such as crop farming (Taylor and Reeder, 2020), aquaculture (Miranda et al., 2018), livestock (Landers et al., 2012). Besides, since the discovery of penicillin by Alexander Fleming, in 1929, antibiotic evolutions are happening constantly for both natural and synthesized antibiotics (Kraemer et al., 2019). Therefore, antibiotics are recently considered as emerging pollutants (Gomes et al., 2020). Much attention has been laid on the antibiotic for few decades, especially along with the increasing consumptions of developing countries where the usage of antibiotic is nearly free (Pokharel et al., 2020). However, the consumption of antibiotics in animal husbandry is indeed much higher than in human medicine (Polianciuc et al., 2020) (see Fig. 1-3); therefore, it is thought that antibiotic pollution in developing countries is ongoing vehemently.

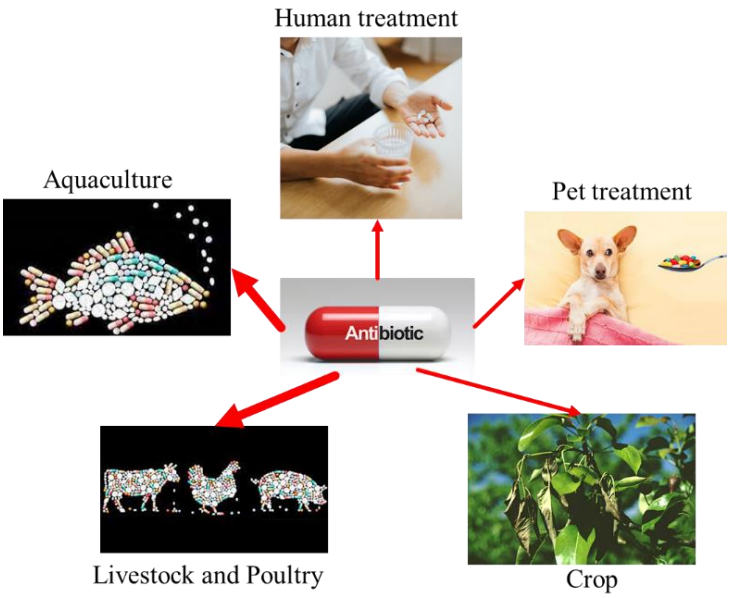

Fig. 1-3. Main applications of antibiotics (figures derived from internet)

In terms of antibiotic characters, it is serious because between 40–90% of the administered antibiotic concentrations are excreted in the feces and urine in the active form into the environment (Polianciuc et al., 2020). For example, those mounts are 90% (Janecko et al., 2016) and 75% (Xu et al., 2021) for fluoroquinolone and tetracyclines classes, respectively. In addition, wastewater treatment plants are not designed for treating sufficiently the wastewater containing antibiotics (53%–78% removed) (Wang et al., 2020). With all of the above-mentioned issues, antibiotics are a heavy burden for the economy, society, and environment (Polianciuc et al., 2020). Fig. 1-4 indicates the constant release of antibiotics due to the need for human treatment and production process under bacterial resistance issues.

So far, among contaminants in the ecosystem, cyanotoxins are considered as the most classical toxicants presenting in the earth 3.5 billion years ago (Francis, 1887) which are produced by cyanobacteria, and therefore they are one of the most natural poisonous groups (Ilieva et al., 2019). Cyanotoxins is a general name of toxic metabolites produced by various cyanobacterial species. Well-known cyanotoxins from specific cyanobacteria, including action mechanisms have been well-reviewed by Zanchett and Oliveira-Filho (2013). In brief, the information of key species producing specific cyanotoxins was shown in Table 1-1.

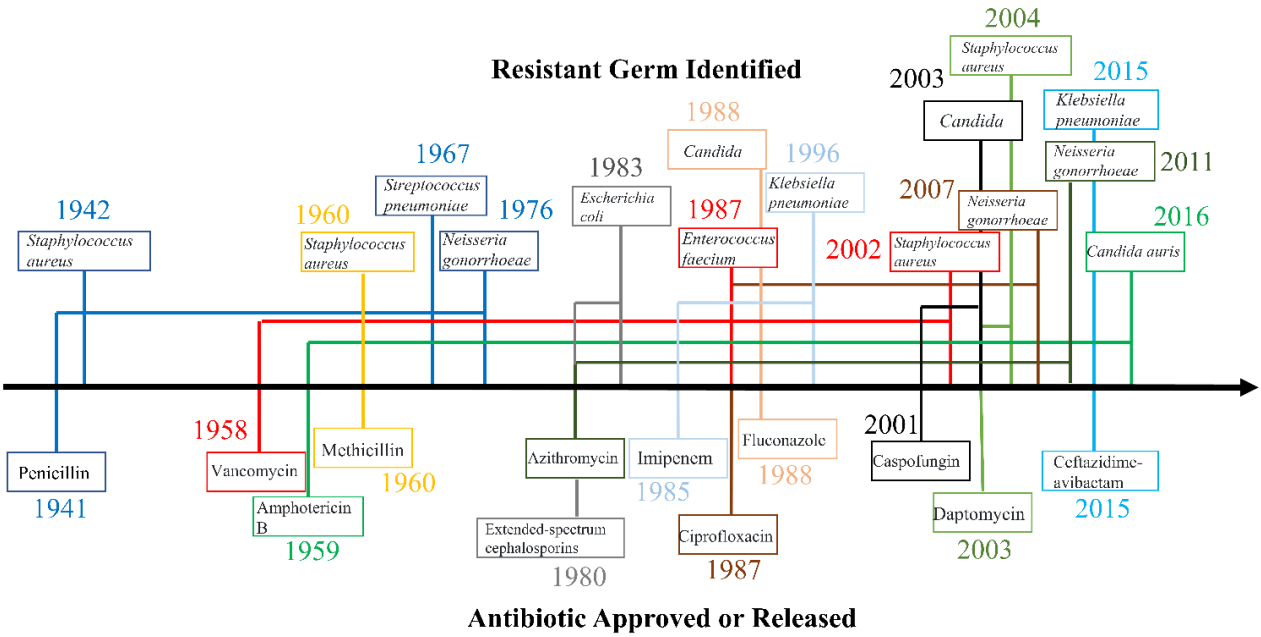

Fig. 1-4. A brief history of resistance and antibiotics (CDC, 2020)

Table 1-1. Key cyanobacterial species producing specific cyanotoxins reported in the meta-analysis (full information is found in Zanchett and Oliveira-Filho (2013))

Cyanotoxins	Name of producers	References
Hepatotoxins		
Microcystins	*Microcystis, Anabaena, Planktothrix*	Mulvena et al., 2012
Nodularins	*Nodularia*	Pearson et al., 2010
Neurotoxins		
Anatoxin-a	*Anabaena, Aphanizomenon, Planktothrix*	Ferrão-Filho, 2009
Saxitoxins	*Anabaena circinalis, Aphanizomenon sp., Aphanizomenon gracile, Cylindrospermopsis*	Bláha et al., 2009
Dermatotoxins		
Lyngbyatoxin-a	*Lyngbya*	Chorus and Bartram, 1999
Lipopolysaccharides	Cyanobacteria in general	Stewart et al., 2006

Cyanotoxins have been received a lot of attention because they are the metabolites of natural organisms whose role is the part of nature which can be seen worldwide in environmental matrices, from freshwater, marine, and even terrestrial habitats (Zanchett and Oliveira-Filho, 2013). The fact that more than 40% of freshwater lakes and reservoirs in the world are known as eutrophic conditions (Chorus and Bartram, 1999); however, they are also the main resources of water supply for humans as well as natural habitats for various creatures (Cooke et al., 2005). Cultural eutrophication is generated mainly by human activities, such as domestic, industrial, and agricultural wastes (Withers et al., 2014; Schneider et al., 2019) which is, therefore, favorable for the development of toxic cyanobacteria (Rastogi et al., 2015). Due to being a part of nature, cyanotoxins can be found over the world as shown in Fig. 1-4.

Fig. 1-5. Country distributions of cyanobacteria (Buratti et al., 2017)

1.1.3. The risks of antibiotics and cyanotoxins on the ecosystems

Over three decades ago, antibiotics do not nearly receive much attention as the detrimental pollutants from ecotoxicologists as well as ecologies because they are thought to be safe for eukaryotes (Almeida et al., 2021). After that, several early studies are performed to investigate the toxic levels of commonly used antibiotics to sensitive organisms, e.g. *Daphnia magna* (Dojmi di Delupis et al., 1992; Wollenberger et al., 2000). Recently, intensive knowledge of the potential risks of antibiotics on individuals, populations, and communities are basically obtained; however, there are still many aspects of antibiotic toxicities that are unsolved and therefore need to be more considered (González-Pérez et al., 2016; Lu et al., 2019; Motiei et al., 2020). Especially, indirect and direct mechanisms of

the antibiotic effects on natural eukaryotes (for example *D. magna*) have been currently discovered (Gorokhova et al., 2015; Aderemi et al., 2018; Bownik et al., 2019; Akbar et al., 2020; Motiei et al., 2020). In practical ecosystems, antibiotics cause disturbances to the cycle of nitrogen (DeVries et al., 2015) and carbon (Wepking et al., 2019) via the extermination of microorganisms responsible for those cycles. Illumination for this was designed as Fig. 1-5. Besides, we well know that those fundamental cycles are considered as the root for ecosystem existence (Zaehle, 2013) and therefore this is how antibiotics exert damages for ecosystems. In humans, regardless of uptaking antibiotics as passive patterns via antibiotic-polluted drinking water (Ben et al., 2020) or consumption of antibiotic-accumulated foods (Pan and Chu, 2017), or as active patterns through oral and intravenous treatments (Hagan et al., 2019), antibiotics can affect the gut microbiome and perhaps lead to the impairment of antibody response (Hagan et al., 2019) and the improvement of bacterial resistance (Iwu et al., 2020). The potential antibiotic effects for humans were reported briefly in Table 1-2 .

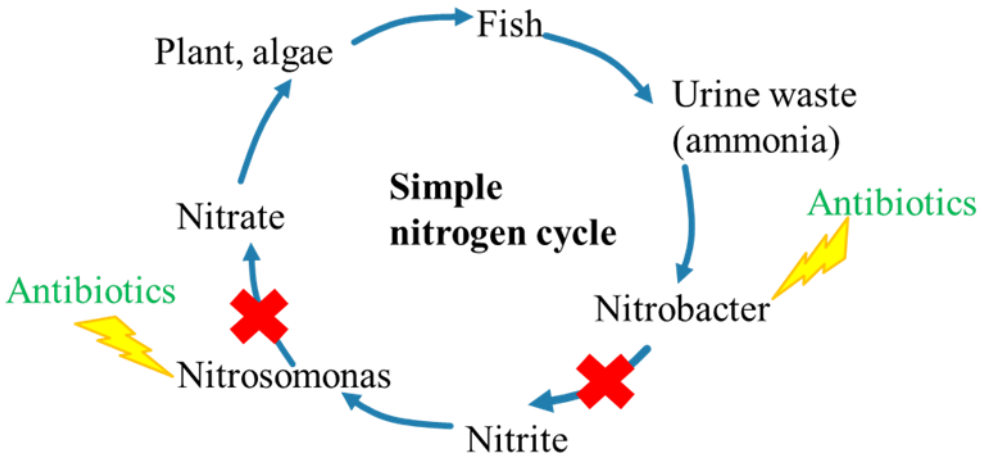

Fig. 1-6. Antibiotics change nitrogen cycles in the ecosystem.
(https://users.vcnet.com/rrenshaw/dan.html)

Table 1-2. The potential effects of antibiotics on human health

Reasons	Consequences	References
Treatment process	Diarrhea , an upset stomach, and nausea, change of heat rate	www.medicalnewstoday.com
Drinking water	The impairment of antibody response	Hagan et al., 2019
Drinking water	Bacterial resistance	Iwu et al., 2020

In terms of cyanotoxins, it is most important to mention that cyanotoxins are serious risks for living organisms because of their high toxicity. Especially, in the events of high biomass blooms, regardless of toxic or nontoxic species, cyanobacteria can exert oxygen depletion (hypoxia and anoxia) in the water environments, resulting in the suppressions of trophic levels (Paerl and Otten, 2013). Cyanotoxin mechanisms of action especially in mammals can be found in the review of Zanchett and Oliveira-Filho (2013). The most abundant and highly toxic cyanotoxin group are microcystins (MCs) (Díez-Quijada et al., 2019), which require additional attention, not only for their ability to cause acute poisonings, such as gastrointestinal malaise, muscle weakness, nausea, vomiting, diarrhea, cough, sore throat, rash, and liver damage due to immersion in the water body experiencing the cyanobacterial bloom for few hours (Giannuzzi et al., 2011; Trevino-Garrison et al., 2015) but also for their ability to initiate liver cancer (tumor promotor) through chronic exposure at a low MCs concentration (Drobac et al, 2013). Specifically, from 1800 to 2010, 115 acute human poisoning cases including mortality and morbidity have been reported around the world (Wood, 2016). Severe cases of human and mamal poisonings were colleted in Table 1-3. From the point of view of ecotoxicity, cyanotoxin exerts effects on whole trophic levels, including bacteria (Adamski, et al., 2019), algae (Pinheiro et al., 2016), aquatic plants (Ujvárosi et al., 2019), freshwater clam (Pham et al., 2015), fish (Zanchett and Oliveira-Filho, 2013), with emphasis on grazers zooplankton (Dao et al., 2013; Bednarska and Slusarczyk, 2013; Nguyen et al., 2020). Besides, cyanotoxin can induce adversely maternal and transgenerational effects for several zooplanktonic species (Dao et al., 2010, 2018). Nevertheless, cyanotoxin magnitude can be increased for aquatic organisms, such as gastropod pulmonate *Lymnaea stagnalis* and freshwater clam *Corbicula leana* P. in relation to bioaccumulated toxic secondary metabolites in the body (Gérard et al., 2005; Pham et al., 2016). Together with directly using drinking water contaminated by cyanobacterial bloom events (Falconer et al., 2005), bioaccumulation of toxic secondary metabolites across the food webs generates an important pathway for humans to expose to cyanotoxin (Flores et al., 2018).

Table 1-3. Several well-known poisonings of cyanotoxins for humans and mammals.

	Reasons	**Consequences**	**Location**	**Year**	**References**
Human	Drinking water	88 deaths	Brazil	1988	Teixeira et al., 1993
Human	Drinking water	150 people with liver and kidney damage	Australia	1990	Sanseverino et al., 2017
Human	Treatment process	50 deaths	Brazil	1996	Jochimsen et al., 1998
Human	Drinking water	100 deaths	Kenya	-	Wood, 2016
Elephant	Drinking water	330 deaths	Botswana	2020	https://www.bbc.com/news/world-africa-54234396

1.2. The aims and study flow of the doctoral dissertation

The main objectives of the doctoral dissertation are to investigate the new and interesting aspects of the potential risks of various antibiotics and cyanotoxins to sensitive organisms as shown in Fig. 1-7. However, it is necessary to declare that in this thesis, we paid more attention to antibiotic ecotoxicology because antibiotics are our main concerns as an emerging pollutant and many gaps remain for mining in our laboratory conditions.

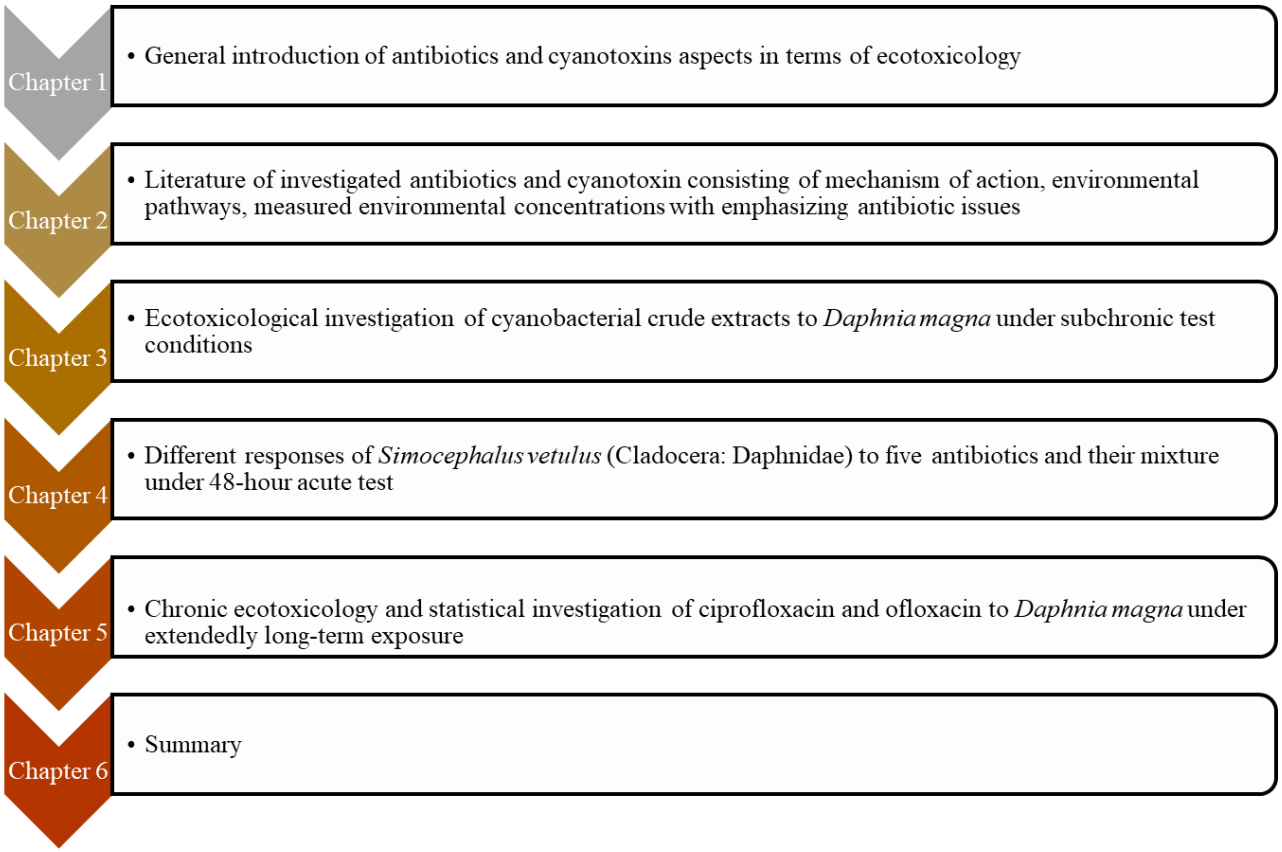

Fig. 1-7. Main contents of the doctoral dissertation

We declare herein that chapters 3, 4, and 5 of the doctoral dissertation represent a published paper, a submitted manuscript, and a preparing manuscript, respectively. For chapter 6, the content is in further development and considered for publication in the future.

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2. REVIEWS OF ANTIBIOTICS AND CYANOTOXINS

2.1. Mechanisms of action

In this study, we used four kinds of antibiotics belonging to fluoroquinolones (FQs) (Fig. 2-1A) and a kind of tetracyclines (TCs), namely tetracycline antibiotic (TET) (Fig. 2-1B). FQs are synthesized antibiotics; therefore, specific antibiotics were generated by substituting "R_n" groups in the FQ structure depending on specific purposes in bacterial infection treatments (Peterson, 2001). Like FQs, TCs can be modified to meet the specific bacterial treatments; however, TCs can be natural (original forms), semi-synthesized, or fully synthesized antibiotics (Liu and Myers, 2016). The importance of each group and optimal substitutions in the TCs molecule can be clarified by Chopra and Roberts (2001), whereas those details for FQs are found in (Gootz and Brighty, 1996).

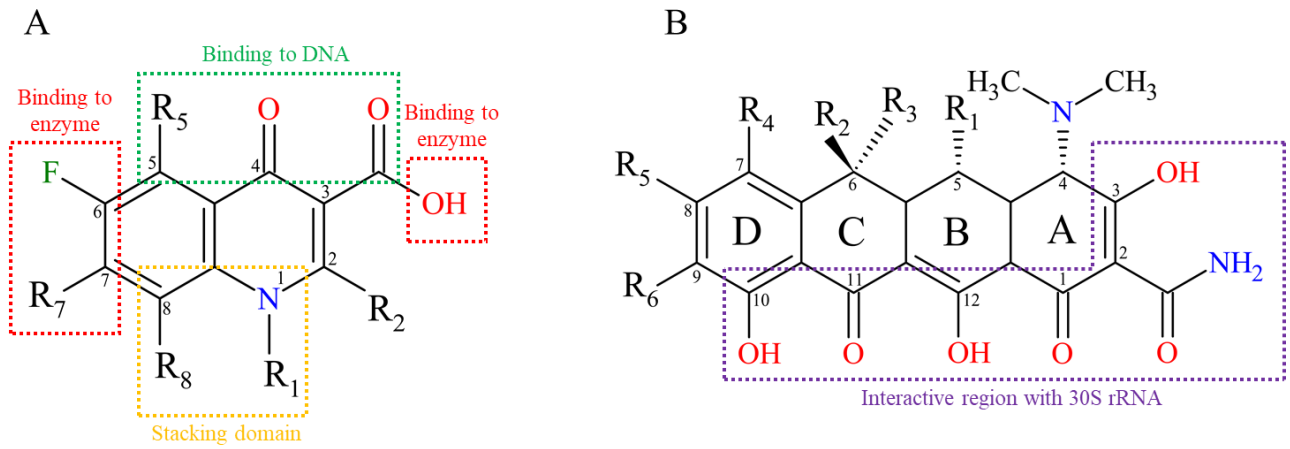

Fig. 2-1. Structure-activity relationships of fluoroquinolones (A) and tetracyclines (B) antibiotic. **Fig. 2-1(A)** is adapted from Vallet (2012) and Bush et al., (2020), and **Fig. 2-1(B)** is adapted from Fuoco (2016).

2.1.1. Fluoroquinolones mechanism of action

For FQs, they are known to inhibit DNA gyrase and topoisomerase IV which together belong to type-II topoisomerase or bacteria. Specifically, FQs form a ternary complex between the DNA, the enzyme (DNA gyrase and topoisomerase IV), and stacked FQs resulting in the blockage of the replication progression, DNA lesions, and finally to bacterial death. FQs interact with the *GyrA* subunit of the DNA gyrase, and the *ParC* subunit of the topoisomerase IV. However, at the same time, the main target in Gram-positive bacteria is topoisomerase IV, whereas in Gram-negative bacteria it is DNA gyrase (Vallet, 2012).

2.1.2. Tetracyclines mechanism of action

TCs inhibit gram-positive and gram-negative bacteria growth by inhibiting translation. Specifically, during translation, it binds to the 30S ribosomal subunit (at A site) where is also the receptor of the

amino-acyl tRNA; therefore, the translation process can not naturally happen. It also binds to some extent to the 50S ribosomal subunit. This binding is reversible. Additionally, it may alter the cytoplasmic membrane of bacteria causing leakage of intracellular contents, such as nucleotides, from the cell (https://go.drugbank.com/drugs/DB00759; Fuoco, 2012; Nguyen et al., 2014).

2.1.3. Cyanotoxin mechanism of action

Unlike antibiotics which mainly act on prokaryotic cells (i.e. bacteria), cyanotoxins can act directly on eukaryotic species, for example, mammals as well as humans (McLellana and Manderville, 2017). Among cyanotoxins, microcystins (MCs) are known as the most toxic and widest distributed metabolites of cyanobacteria (Bláha et al., 2009). Mechanisms of toxicity of MCs include cellular uptake, interaction with protein phosphatases PP1 and PP2A, cytoskeletal effects, formation of oxidative stress, and induction of apoptosis. An excellent description of the cyanotoxin interaction mechanism with mammalian cells can be found in (Zanchett and Oliveira-Filho, 2013; McLellana and Manderville, 2017).

2.2. Environmental pathways of antibiotics and cyanotoxins

2.2.1. Environmental pathways of antibiotics

It is noteworthy to mention that FQs and TCs are the most intensive and widest applied antibiotics for human, animal treatment as well as the production process (Zalewska et al., 2021). Therefore, it is not surprising that those antibiotics can reach the environment under active residues with extremely high concentrations (see section 2.2.2). The potential environmental pathways of antibiotics were shown in Fig. 2-2.

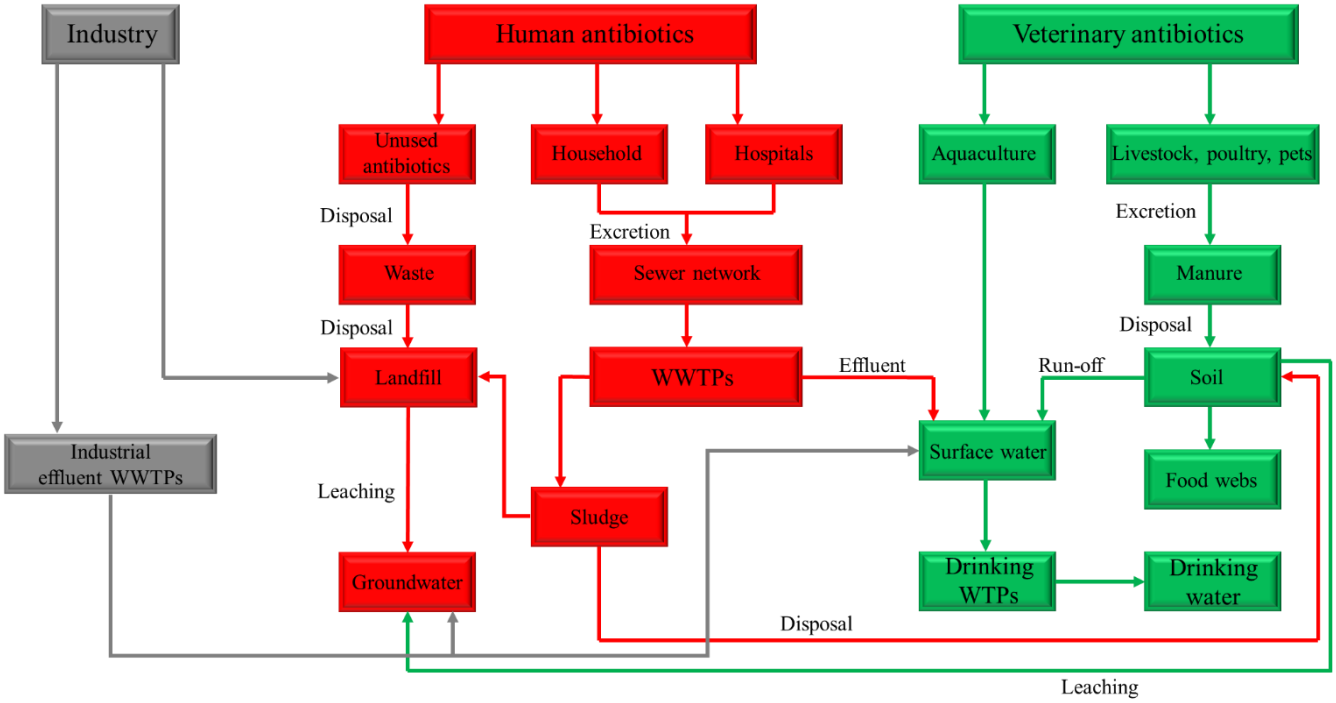

Fig. 2-2. Environmental pathways of antibiotics from application to receiving water body (adapted from Faleye et al., 2018).

Among those pathways, the effluent from pharmaceutical industrial zones is the massive source to convey antibiotic residues to environmental compartments (Thai et al., 2018). For example, up to 31000 µg L^{-1} of ciprofloxacin (CFX) was detected in the surface water around pharmaceutical manufacturers in India, even though the wastewater treatment facility was working (Larsson et al., 2007). Moreover, the developing countries are thought to be in the most serious circumstances of antibiotic pollution, because the usage of antibiotics for human treatment and production process is not managed by the government (Chokshi et al., 2019) and conventional WWTPs (or even none of the conventional WWTPs) are not proper to remove efficiently antibiotic residues (Booth et al., 2020). However, the developing countries are also reported to be the biggest manufacturers as well as biggest consumers of antibiotics in the world (Thai et al., 2018).

2.2.2. Cyanotoxins in the aquatic environment

Various cyanotoxins can be detected as extracellular metabolites during bloom events of toxic cyanobacteria (Keliri et al., 2021) depending on the specific cyanotoxin-producing cyanobacteria included (Bukowska et al., 2017; Christophoridis et al., 2018). Chemically, the cyanotoxins are divided into three main groups consisting of cyclic peptides (MCs and nodularins), alkaloids (anatoxin-a, anatoxin-a(s), saxitoxins, cylindrospermopsin, aplysiatoxin, lyngbiatoxin-a), and lipopolysaccharides (Rastogi et al., 2015). Almost all lakes and reservoirs, estuarine, coastal lagoonal estuaries, and coastal waters located in continents can occur cyanobacterial bloom events depending on the favorable conditions for photosynthesis, such as sunlight, carbon dioxide, and enriched nutrient (i.e. eutrophication) (Chislock et al., 2013; Paerl and Otten, 2013). Besides, global warming plays an

important role to improve cyanobacterial development and potential blooms in the water body, because cyanobacteria are well known to have optimal growth rates at high water temperatures (Paerl and Otten, 2013). Therefore, cyanotoxins detected in cyanobacterial blooms in the tropical zone are likely to broadly distribute, highly diverse, and frequently occur compared to other regions (Mowe et al., 2015). Fig. 2-3 indicates the heavy cyanobacterial bloom containing multiple cyanotoxins in Tri An reservoir, Vietnam.

Fig. 2-3. The heavy cyanobacterial bloom in Tri An reservoir, Vietnam. (a), (b), (c) mean observations of June, September, November (2016), respectively, and (d) means water without bloom (Nguyen et al., 2020).

2.3. Measured environmental residues of antibiotics and cyanotoxins

2.3.1. Measured environmental residues of antibiotics

To demonstrate occurrences of the investigated FQs (ciprofloxacin-CFX, ofloxacin-OFX, gatifloxacin-GFX, delafloxacin-DFX) and TCs represented by tetracycline (TET), in aquatic environmental matrices, we did a meta-analysis of published papers measuring those antibiotics which are integrated from 2010 to 2020. Totally, 100+ papers have been collected which reported investigated antibiotic residues covering all continents of Asia, Europe, America, Oceania, and Africa. Besides, the aquatic environmental matrices have reported the presence of antibiotics consisting of the effluents from pharmaceutical WWTPs (PWWTPs), hospital WWTPs (HWWTPs), livestock and aquaculture effluents (LAEs), municipal WWTPs (MWWTPs), rivers (or streams and canals), and lakes (or reservoirs). Complete data are shown in Table A-1. However, as very huge data was collected for rivers, we, therefore, showed only around 50% of those data prioritizing the high values. Subsequently, Fig. 2-4 indicated the comparison of CFX and OFX residues in various environmental matrices with full collected data, concentrations were showed as natural logarithms due to high variance. Finally, the summary of the whole data was shown in Table 2-1.

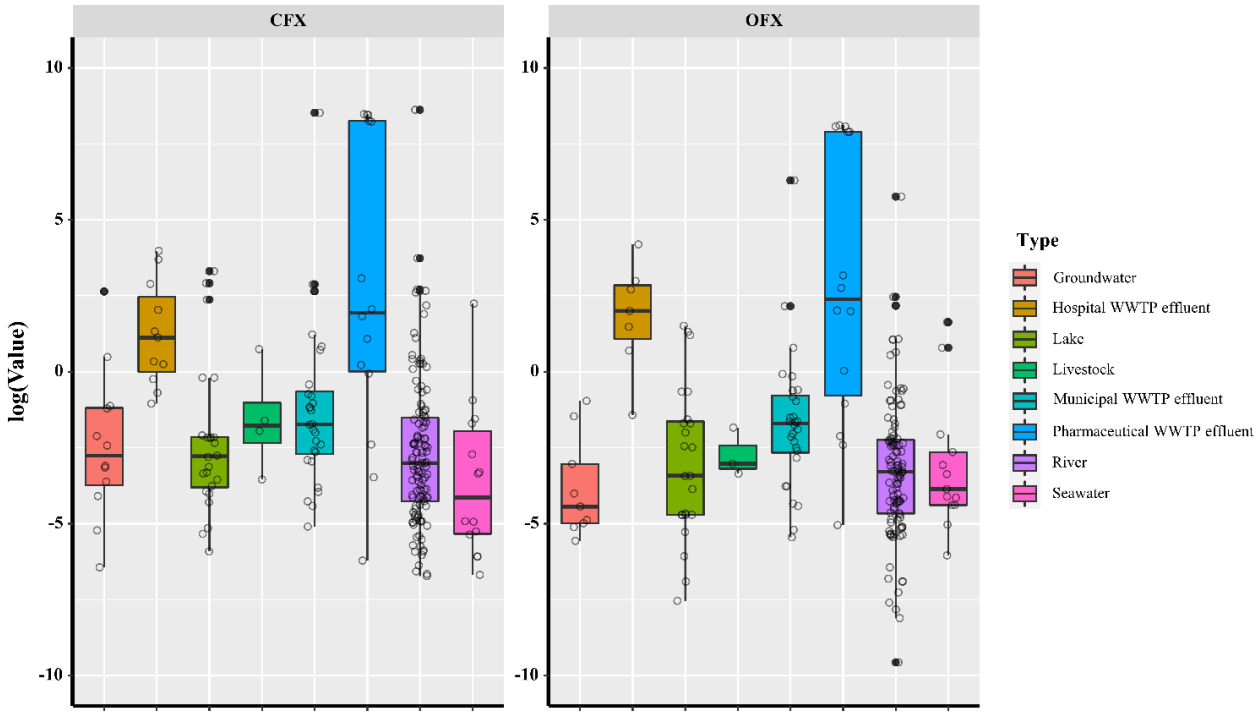

Fig. 2-4. Collected data of ciprofloxacin and ofloxacin residues in various environmental matrices.

Table 2-1. Summary of collected data from 100+ papers of CFX and OFX residuals in various environmental matrices.

	CFX			
	Min	**Max**	**Median**	**Mean**
Groundwater	0.0016	14.00	0.0665	1.382
Hospital WWTP effluent	0.35	53.30	3.08	11.844
Lake	0.0027	27.31	0.062	2.462
Livestock	0.0288	2.10	0.171	0.6177
Municipal WWTP effluent	0.0061	5015.60	0.1769	180.684
Pharmaceutical WWTP effluent	0.002	4800	7.02	1561.49
River	0.0012	5528.90	0.049	46.76
Seawater	0.00125	9.41	0.021	0.74
	OFX			
	Min	**Max**	**Median**	**Mean**
Groundwater	0.00382	0.3822	0.0118	0.0794
Hospital WWTP effluent	0.24	66.0	7.4	16.4
Lake	0.00053	4.526	0.0326	0.593
Livestock	0.0349	0.1590	0.0484	0.0808
Municipal WWTP effluent	0.0043	542.45	0.1814	19.963
Pharmaceutical WWTP effluent	0.0064	3330	11.603	1080.42
River	0.00007	318.1	0.0372	3.331
Seawater	0.0024	5.1	0.021	0.5897

It is very clear that collected data for CFX and OFX is dominated followed by TET, and finally GFX. There is no data available for DFX because DFX is a newly approved antibiotic. For more information on this issue, see section 4. The collected data indicates that antibiotic residues of CFX and OFX in

PWWTPs and surrounding receiving water bodies are highest concentrations followed by HWWTPs. In general, the trends and quantities of CFX and OFX residues are relatively similar. On the other hand, TET residues are also considerably high in PWWTPs compared to other sources; however, collected data for TET and GFX is very few to correctly evaluate and therefore their data need to be more collected.

2.3.2. Measured environmental concentrations of cyanotoxins in cyanobacterial blooms

During the cyanobacterial bloom, Pawlik-Skowronska et al. (2016) measured the intracellular microcystins and anatoxin-a of surface water containing cyanobacteria (mixture of biomass) in four reservoirs in Poland. The results showed that MCs (MC-RR, -LA, -LW, and -LF) were detected prominently in water samples of four reservoirs (total MC concentration up to 25.3 μg L^{-1}) comparing to anatoxin-a which was insignificant (0.59 μg L^{-1}). Recently, Trung et al. (2018) measured the cyanotoxins during the cyanobacterial blooms in duck–fish ponds in Tra Vinh province, Vietnam. The results showed that microcystins consisting of MC-RR and MC-LC dominated in total cyanotoxins of collected samples. The total concentrations of MCs were up to 91,721 μg L^{-1} in water samples and up to 4033 μg g^{-1} DW in biomass samples.

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3. THE ECOTOXICOLOGICAL INVESTIGATION OF CYANOBACTERIAL CRUDE EXTRACTS TO *DAPHNIA MAGNA* UNDER SUBCHRONIC TEST CONDITIONS

3.1. Introduction

It is well known that lakes and reservoirs are one of the major potable water resources for humans and other living beings and are habitats for a wide variety of species (Cooke et al., 2005). Unfortunately, more than 40% of lakes and reservoirs in the world are in eutrophic conditions, which is favorable for cyanobacterial bloom events (Chorus and Bartram, 1999). Cyanobacteria are harmful to aquatic organisms in water bodies due to their ability to produce a variety of toxic secondary metabolites, particularly during their mass development (Sivonen, 1996). Therefore, cyanobacterial blooms are the major concern of human and ecological health (Bláha et al., 2009). In general, cyanobacteria can induce numerous negative effects on zooplankton including inhibition of the feeding rate, reduction in survival rates, reproduction, and somatic growth (Ferrao-Filho et al., 2000; Dao et al., 2010). However, zooplanktonic species can respond differently to toxins or other bioactive compounds produced by cyanobacteria (Ferrao-Filho et al., 2000).

Among the toxic secondary metabolites produced by cyanobacteria, microcystins (MCs) are the most investigated ones (Guzmán-Guillén et al., 2017), particularly related to zooplankton (e.g. *Daphnia* spp.) due to their toxicities and ubiquitous distribution (Díez-Quijada et al., 2019). Therefore, the adverse effects of the microcystin-producing strain of cyanobacteria on zooplankton have been extensively investigated (Liang et al., 2017). Although the microcystin-free strain does not contain known microcystin toxins, its negative effects on *Daphnia magna* (*D. magna*) were reported in a few studies (e.g. Lurling, 2003; Hulot et al., 2012). A number of previous studies often used living cells (Ferrao-Filho et al., 2000; Lurling, 2003; Dionisio Pires et al., 2005; da Costa et al., 2013; Pham et al., 2015a), purified MCs (Chen et al., 2005; Ortiz-Rodríguez et al., 2010; Ortiz-Rodríguez et al., 2012; Hulot et al., 2012), or cyanobacterial crude extracts (CCEs) (Pietsch et al., 2001; Dao et al., 2010; Hulot et al., 2012; Dao et al., 2013a; Pham et al., 2016; Pawlik-Skowrońska et al., 2019; Toporowska et al., 2020) in toxicity tests. In fact, water from cyanobacterial blooms contains not only MC but also a mixture of multiple substances which can cause unpredictable effects on zooplankton (e.g. *Daphnia* spp.) (Okumura et al., 2007). Therefore, the use of CCEs as the toxicant to evaluate the harmful status of water bodies on aquatic organisms during the events of cyanobacterial blooms is highly recommended (Pietsch et al., 2001). Our current knowledge about the response of *D. magna* (e.g. life-history characteristics and feeding rate) to the microcystin-free crude extract (NCCE) of *Pseudanabaena* sp. is limited.

In Vietnam, toxicities of the microcystin-containing crude extract (MCCE) of *Microcystis* spp. sampled from Dau Tieng Reservoir, which is the biggest irrigation reservoir of Vietnam and potable water resource in southern Vietnam (Pham et al., 2015b), has been not completely evaluated. Although a few notable great attempts (e.g. Dao et al., 2013b; Dao et al., 2014; Pham and Ngo, 2017; Pham et al., 2017; Dao et al., 2018) have indicated the adverse effects of cyanobacterial blooms (including the *Microcystis* spp. living cells and MCCE) collected from Dau Tieng Reservoir on several model organisms, the life-history traits and feeding rate of *D. magna* at an early stage of development, a highly important stage that can influence the whole lifespan of organisms (e.g. reproduction) (Van Leeuwen et al., 1985), have not been reported. As a continuation of previous

publications, the aim of this study was, therefore, to investigate the subchronic toxicity of MCCE and NCCE on the life-history traits and feeding rate of *D. magna* at its early stage of development.

3.2. Materials and methods

3.2.1. Test organisms

Samples of *D. magna* obtained from Microbiotests Inc, Belgium was used for the toxicity test. These organisms were raised in the ISO medium (Dao et al., 2010) and fed by a mixture of viable green algae *Chlorella* sp. and *Scenedesmus* sp., which were cultivated in the COMBO medium (Kilham et al., 1998) with continuous aeration. Both *D. magna* and green algae were maintained at a temperature of 25 ± 1 °C and 12/12 h of light/dark cycle.

3.2.2. Cyanobacterial crude extract preparation

Biomasses extracted from *Microcystis* spp. and *Pseudanabaena* sp. blooms were used as cyanobacterial materials for the toxicity tests. The scum of cyanobacteria during the events of blooms, mainly *Microcystis* spp., was collected from Dau Tieng Reservoir located around 85 km from northwest Ho Chi Minh City, Vietnam. The cyanobacterial sample was dried under sunlight and kept at −20 °C prior to the extraction for the test. The microcystin-free cyanobacterial strain (*Pseudanabaena* sp.) isolated from the Dau Tieng Reservoir (Pham et al., 2015b) was cultured in a Z8 medium and harvested onto GF/C glass fiber filters (Whatman, Kent, England). The filters containing *Pseudanabaena* sp. were dried at 45 °C and kept at −20 °C until the experiment. CCEs were prepared as previously reported by Pietsch et al. (2001): 2g dry weight (DW) of the bloom material or *Pseudanabaena* sp. isolate were put into distilled water, frozen at −70 °C, and then thawed at room temperature. After the materials were thawed completely, they were sonicated for 3 minutes. This freeze-thaw-sonicate cycle was repeated five times and then the samples were centrifuged at 2000 × g for 10 minutes to remove cell debris. The supernatants were collected and kept at −20 °C until the toxicity experiments. For analyzing the MC content, the concentration of 8 g L^{-1} of CCEs (w/v) was prepared. Subsamples of the CCE supernatant were used for MC analysis as previously reported by Pham et al. (2015b): 100 mL of the supernatants were centrifuged at 6000 × g at 4 °C for 15 min. The supernatants were collected, dried completely, and re-dissolved in 500 µL of 100% MeOH. The samples were analyzed by the High-performance liquid chromatography (HPLC) system with the Ultraviolet-visible photodiode array detector (Shimadzu 10A series, Kyoto, Japan). MC-RR, MC-LR, and MC-YR (Wako, Osaka, Japan) were used as standards. The HPLC analysis showed that CCE from natural cyanobacterial blooms contained three MC congeners, namely, MC-RR, MC-LR, and MC-YR, at a total concentration of 670 µg g^{-1} DW (Pham et al., 2015b), whereas MCs were not detected in the extract of *Pseudanabaena* sp.

3.2.3. Ecotoxicological experiments

Experiment on life-history traits of *D. magna*

The ecotoxicological test on life-history traits of *D. magna* was conducted according to Dao et al. (2010). The experiments consisted of the control and CCE exposures. The control was run in which

D. magna was raised in ISO medium without any cyanobacterial extract addition. For CCE exposures, MCCE was added into medium to reach desired concentrations (1, 10, and 50 μgMC L^{-1}), which (i.e. those MC concentrations) are often found in the natural environment during cyanobacterial blooms (Chorus and Bartram, 1999). NCCE was used at the same concentrations (i.e. the injected volume) of MCCE, hereafter referred to as M1, M10, M50 for MCCE, and N1, N10, N50 for NCCE, respectively. For each exposure, fifteen neonates (< 24 h old) were randomly collected and individually transferred into 50 mL beakers containing 30 mL ISO medium. The organisms were fed with *Scenedesmus* sp. at a concentration of 1 mg carbon source L^{-1} (approximately 140,000 cells mL^{-1}) during the test. Test medium and food were renewed simultaneously every two days. Besides, the pH and dissolved oxygen in each culture beaker were measured for fresh and spent medium. The measured pH and dissolved oxygen (mg L^{-1}) in the culture medium were within a range of 7.1 to 7.6 and from 7.4 to 7.7, respectively, and the test lasted for 14 days. During experiments, the life-history trait responses were evaluated based on survival rates, reproduction, and somatic growth. The survival rate of initial maternal *D. magna* was recorded daily. The reproduction endpoints included the number of neonates per female, brood size, time to maturation, time to first reproduced brood, the number of broods per female, and the intrinsic rate of natural increase. Note that the reproduced neonates were daily counted and discarded. The degradation of eggs and neonates represented by dead eggs, dead neonates, and neonate malformation (if any) was recorded when the dead eggs, dead neonates, and neonate malformation were detectable. The intrinsic rate of natural increase (r) was calculated according to the Jackknife procedure (Meyer et al., 1986):

$$\sum_{x=1}^{\max age} e^{-rx} l_x m_x = 1$$

where, l_x is the proportion of individuals surviving to age x, m_x is the number of neonates produced per surviving females at age x and x is in days. As the calculations of the intrinsic rate of natural increase for 14 days are indistinguishable from those for the entire lifespan and due to the great importance of early-stage reproduction (Van Leeuwen et al., 1985), the calculations of the intrinsic rate of natural increase based on 14-day experiments were therefore acceptable. The somatic growth of the surviving individuals of each exposure was determined when the test was completed. Specifically, the body length of each surviving individual was measured from the apex of the helmet to the base of the tail spine under an inverted microscope (Villarroel et al., 2003).

Experiment on the feeding rate of *D. magna*

The feeding rate experiment was conducted according to the description of Ferrando et al. (1993) with minor modifications. Briefly, three concentrations (as same as the life-history trait experiment) of CCEs (MCCE and NCCE), were used for the feeding rate study and each concentration consisted of five replicates. Another exposure was also implemented as the control in which *D. magna* was incubated in a medium without CCEs. One additional replicate without *D. magna* was used for calculating the correction factor. The experiment was conducted in 50 mL glass beakers containing 50 mL of ISO medium and 10 of *D. magna* (< 24 h old) without green algae as food. The beakers were placed at 25±1 °C under dark and static conditions. After 24 hours, *Scenedesmus* sp. was added into each beaker to reach the desired concentration of 10^5 cells mL^{-1} and the experiment lasted for 5

hours afterward. At the end of the experiment, *D. magna* was removed and the density of *Scenedesmus* sp. in the beakers was estimated by using a hemocytometer counting chamber under an inverted microscope. For the calculations of filtration rate (µL individual^{-1} h^{-1}) (FR) and ingestion rate (cells individual^{-1} h^{-1}) (IR), the equations of Gauld (1951) were used:

$$A = \frac{\ln C'_0 - \ln C'_t}{t}$$

$$IR = FR\sqrt{C_0 C_t}$$

$$FR = \frac{V(\ln C_0 - \ln C_t)}{nt} - A$$

where, C_0 and C_t are initial and final algae densities, respectively (cells µL^{-1}), t is time (period of the experiment in hours), and n is the number of *D. magna* in volume V (µL). A correction factor (A) is a change in the initial (C'_0) and final (C'_t) algae densities after time t (in one additional replicate). The expression $\sqrt{C_0 C_t}$ represents the geometric mean of algae density during time t.

3.2.4. Data mining and analysis

All data were presented as mean ± standard deviation except the survival rates. Type I error level was set at *P*-value ≤ .05, which is considered as the statistically significant level to reject the null hypothesis. The statistical analysis was carried out using R software (version 3.4.3) combining with RStudio (version 1.2.5033). One-way analysis of variance (ANOVA) test was applied to detect the significant difference of reproduction, somatic growth as well as the feeding rate of *D. magna* between the control and CCE exposures (the CCE concentrations were assigned as the factor variables), followed by many-to-one comparison Dunnett's test using "multcomp" package. The assumptions of the ANOVA test were confirmed prior to the test. Levene's test (in "car" package) and Shapiro-Wilk test were applied for checking the homogeneity of variances and normality of residuals, respectively. In case the assumptions were not satisfied, the non-parametric Kruskal-Wallis rank-sum test was applied, followed by the non-parametric Wilcoxon rank-sum test for multiple comparisons. The highest correlation between the *D. magna* responses (reproduction endpoints, somatic growth, and feeding rates) and CCE concentrations, if any, was evaluated by running simple linear (or nonlinear) models for each of CCEs with the CCE concentrations assigned as the numeric variables.

3.3. Results

3.3.1. Effects of cyanobacterial crude extracts on life-history traits of *D. magna*

Effects on survival rates

No mortality occurred in the control, M1, N10, N50, and M50 exposures over 14 days of the test, whereas exposures to M10 and N1 decreased the survival rates of *D. magna* slightly at 6.7% and 13.3% of total initial females, respectively.

Effects on reproduction

The number of neonates per female of MCCE and NCCE exposed *D. magna* at all the exposures was significantly higher than the control (Table 3-1). The time to maturation of *D. magna* exposed to both CCEs at all the exposures (except M1 exposure) was significantly earlier than the control (Table 3-1). Subsequently, *D. magna* belonging to all MCCE and NCCE exposures (except M1 exposure) reached their first reproduced brood at significantly sooner days relative to the control *D. magna* (Table 3-1). The number of broods per female in N10, N50, M10, and M50 exposures was significantly more than the control (Table 3-1). Under exposures to M1, M10, M50, and N50, the first brood size was significantly larger than the control, whereas all MCCE and NCCE exposures significantly increased the second brood size compared to the control (Figure 3-1A, 3-1B). The intrinsic rate of natural increase of *D. magna* in all MCCE and NCCE exposures were significantly higher than the control (Table 3-1). Dead eggs, dead neonates, and neonate malformation of the gravid females were observed in the M50 and N50 exposures (represented by Figure 3-2A, 3-2B, 3-2C, and 3-2D as the control), whereas only dead neonates were observed in the M1, M10, N1, and N10 exposures (represented by Figure 3-2B). Among the reproduction endpoints, somatic growth, and feeding rate of *D. magna*, the number of neonates per female analyzed had the highest correlation with the tested concentrations of both CCEs (Figure 3-3). The regression equations were calculated by the nonlinear model (or the quadratic equation) (Figure 3-3).

Table 3-1. The reproduction of *Daphnia magna* exposed to cyanobacterial crude extracts over the 14-day test.

Exp.	The number of neonates per female (ind.)	Time to maturation (d.)	Time to first reproduced brood (d.)	The number of broods per female (br.)	r (day $^{-1}$)
CT	8.9 ± 2.9	9.1 ± 1.4	11.4 ± 1.2	1.8 ± 0.4	0.15 ± 0.03
M1	16.8 ± 4.2a	7.9 ± 1.6	10.6 ± 1.3	2.3 ± 0.6	0.20 ± 0.02c
M10	42.2 ± 8.9c	5.5 ± 0.8c	7.7 ± 0.8c	3.6 ± 0.6c	0.26 ± 0.02c
M50	74.3 ± 13.0c	5.1 ± 0.8c	7.1 ± 0.8c	4.3 ± 0.6c	0.31 ± 0.01c
N1	19.3 ± 5.8b	7.3 ± 0.5b	9.4 ± 0.5c	2.5 ± 0.5	0.20 ± 0.02c
N10	45.3 ± 5.7c	5.3 ± 0.6c	7.3 ± 0.5c	3.7 ± 0.5c	0.27 ± 0.01c
N50	60 ± 20.0c	5.1 ± 1.3c	7.1 ± 1.3c	3.6 ± 0.8c	0.29 ± 0.04c

*Small letters indicate significant differences by the ANOVA test, followed by the many-to-one comparison Dunnett's test ($^{a}P \leq .05$, $^{b}P \leq .01$, $^{c}P \leq .001$); “Exp.”: exposure, “ind.”: individual, “d.”: day, “br.”: brood, “r”: the intrinsic rate of natural increase.

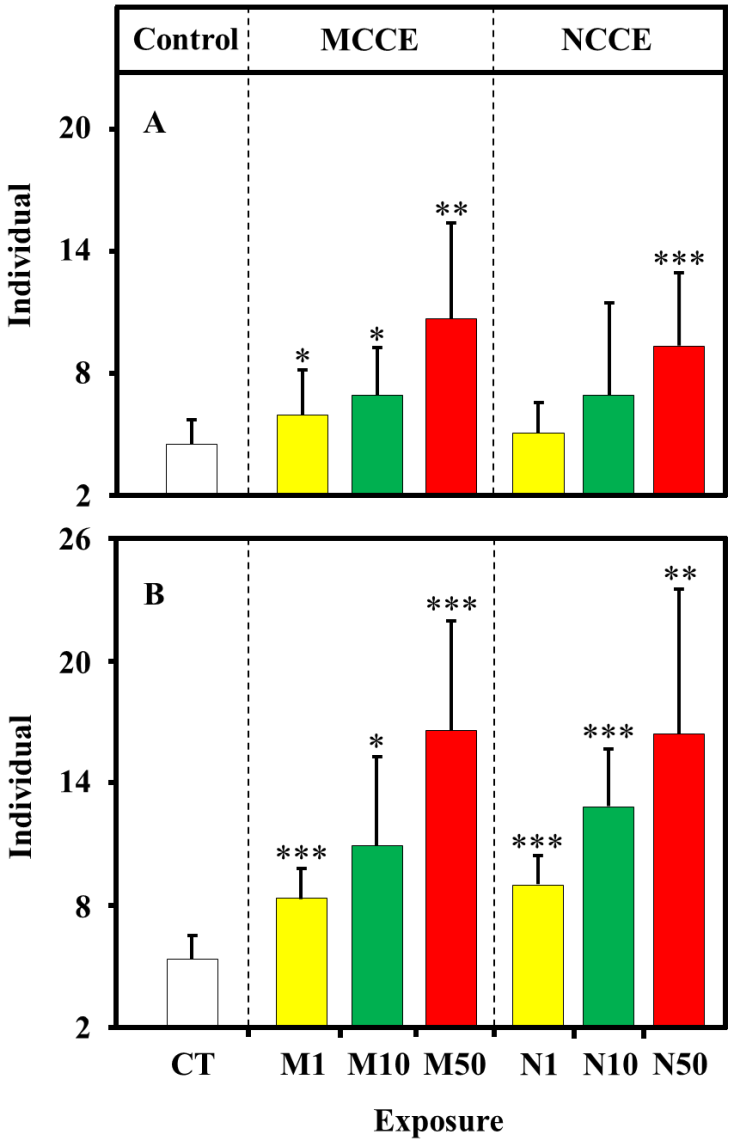

Fig. 3-1. The effects of cyanobacterial crude extracts on the brood size, A: the first brood, B: the second brood. Asterisks indicate significant differences by the Kruskal-Wallis test, followed by the Wilcoxon rank-sum test for multiple comparison (*$P < .05$, **$P < .01$, ***$P < .001$).

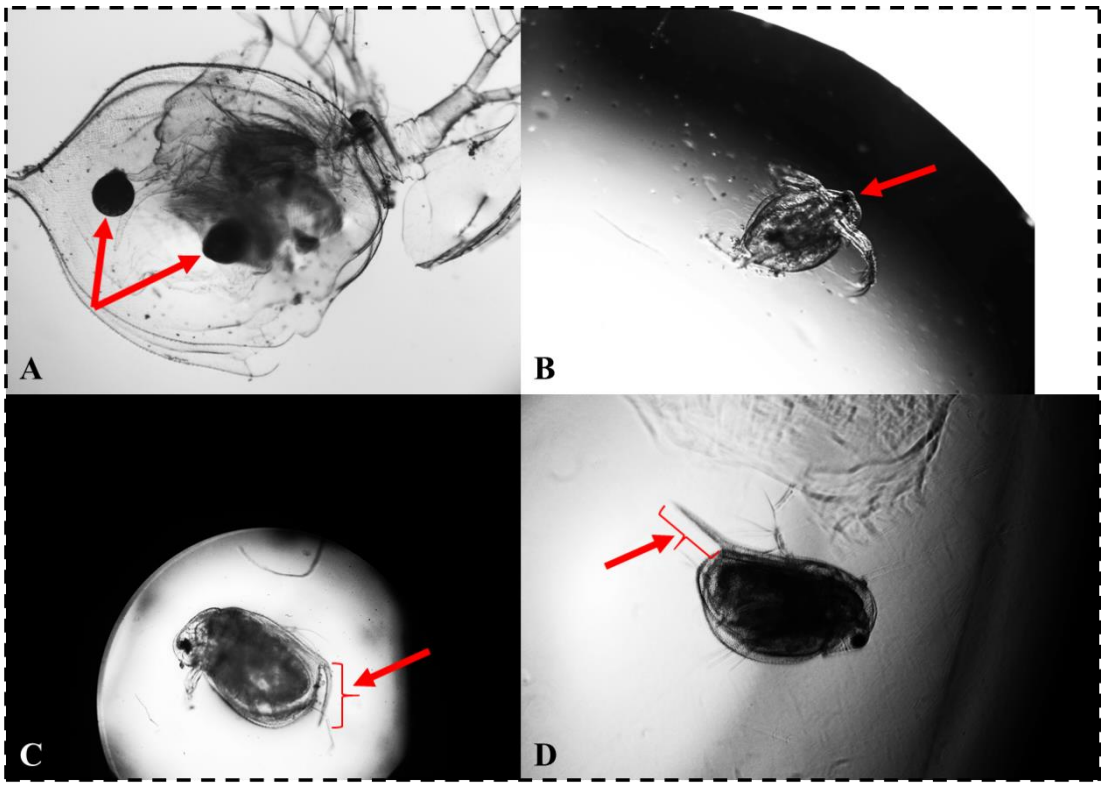

Fig. 3-2. The degradation of eggs and neonates (red arrows) of the *Daphnia magna* gravid females exposed to cyanobacterial crude extracts; A: dead eggs, B: dead neonate, C: malformation of the tail, and D: normal tail of control *Daphnia magna*.

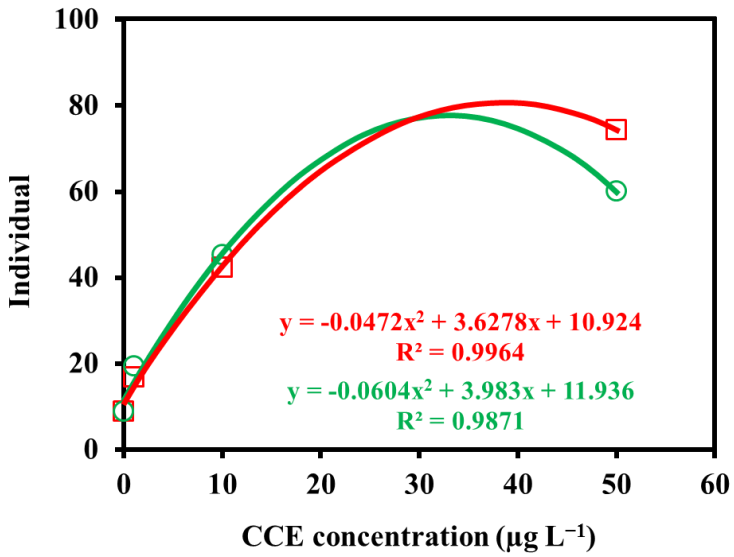

Fig. 3-3. Regression equations and correlation coefficients (R^2) describing the highest correlations between the number of neonates per female (as mean) and CCE concentrations. The red color indicates the microcystin-containing crude extract and the green color indicates the microcystin-free crude extract.

Effects on somatic growth

D. magna body length in all MCCE and NCCE exposures were significantly longer than the control (Figure 3-4).

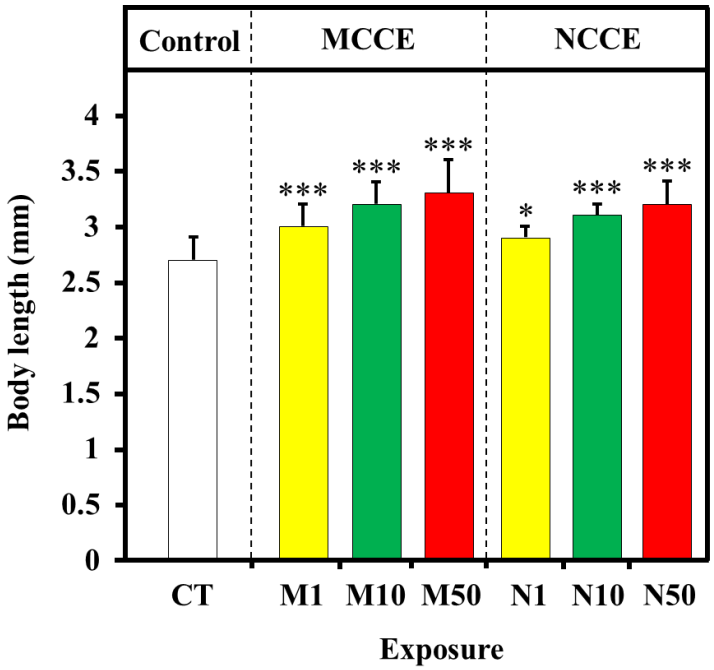

Fig. 3-4. The effects of cyanobacterial crude extracts on somatic growth of *Daphnia magna*. Asterisks indicate significant differences by the Kruskal-Wallis test, followed by the Wilcoxon rank-sum test for multiple comparison (*$P < .05$, **$P < .01$, ***$P < .001$).

3.3.2. Effects of cyanobacterial crude extracts on the feeding rate of *D. magna*

The filtration and ingestion rates of *D. magna* in all MCCE exposures were significantly higher than those in the control, whereas no change in *D. magna* feeding rate in all NCCE exposures was observed relative to the control (Figure 3-5A, 3-5B).

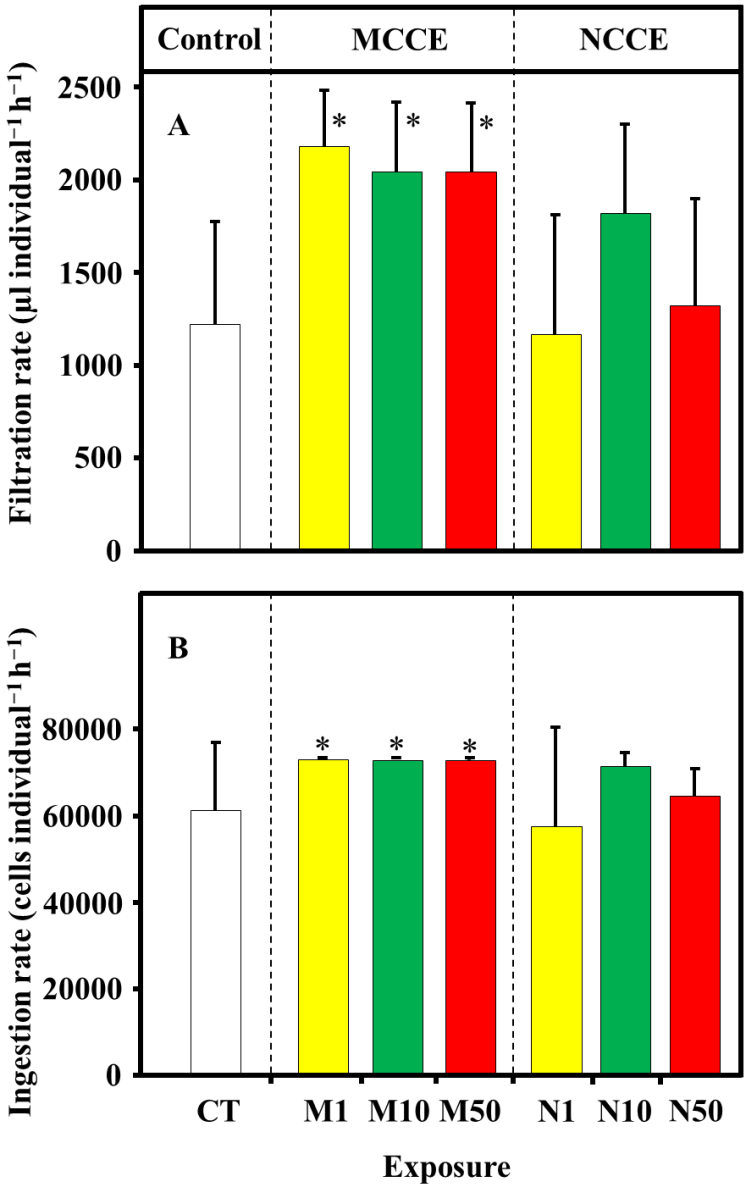

Fig. 3-5. Filtration (A) and ingestion (B) rates of *Daphnia magna* after exposing to cyanobacterial crude extracts. Asterisks indicate significant differences by the Kruskal-Wallis test, followed by the Wilcoxon rank-sum test for multiple comparison (*$P \leq .05$).

3.4. Discussion

D. magna mortality rates under the effects of MCCE and NCCE were insignificant according to OECD (2012), where the regulated survival rate was ≥ 80%. As expected, NCCE could not cause strong adverse effects on survival rates because of the MC-free containing (Ortiz-Rodríguez et al., 2012). As evidenced by Hulot et al. (2012), the survival rate of *D. magna* exposed to NCCE was not significantly decreased over the long-term test. In terms of MCCE effects on survival rates, Dao et al. (2010) reported that MCCE at a low concentration of 5 µgMC L^{-1} decreased the *D. magna* survival rates insignificantly over the 60 days of the test. Moreover, Smutná et al. (2014) pointed out that MCCE at a medium concentration of 9.2 µgMC L^{-1} did not significantly reduce *D. magna* survival rate over 21 days of the test. At a high concentration of 50 µgMC L^{-1}, the harmful effects of MCCE on *D. magna* were likely to be time-dependent. Thus, the adverse effects of MC and/or harmfully

bioactive compounds on *D. magna* over 14 days were not enough to induce mortality. As proof, Dao et al. (2010) revealed that MCCE at a high concentration of 50 µgMC L^{-1} caused high mortality for *D. magna* after the 21st day. In addition, the dissolved MC content in aqueous extracts was likely to be declined as a consequence of the process of adsorption on particulate materials, photodegradation, and biodegradation (Schmidt et al., 2014). Therefore, we suggest that future research should measure the MC concentration in the spent medium (medium after two days of the test) to confirm the remaining MC concentration. Moreover, the toxic effects of MCCE on survival rates were perhaps compensated by multiple substances (considered as the nutrients), and hence MC in a mixture could not cause negative effects on the survival of *D. magna* compared to purified MC (Ortiz-Rodríguez et al., 2012). It was reasonable as a meta-analysis (Smutná et al., 2014) indicated that the toxic effects on survival rates of MC-containing cyanobacterial bloom materials on *D. magna* were decreased significantly depending on the complexity of tested materials with a toxic rank of several substances-containing extract > total aqueous extract > biomass. Our MCCE consisted of a mixture of MC-RR, -LR, and -YR, of which the MC-RR was the dominant congener (Pham et al., 2015b). Thus, MC-RR is likely to induce low toxicities compared to MC-YR or MC-LR by a toxic rank of MC-YR > MC-LR > MC-RR (Puerto et al., 2009). Besides, Campos et al. (2014) gave the proof of multixenobiotic resistance mechanism of *D. magna* which could recognize a wide variety of toxicants (e.g. MC and/or harmfully bioactive compounds in MCCE) and keep them in cells at low levels (Epel et al., 2008).

Our results of reproduction-stimulated effects were in good agreement with Dao et al. (2010), of which *D. magna* was exposed to MCCE at concentrations of 5 and 50 µgMC L^{-1}. However, higher levels of stimulation on the reproduction in our study compared to the previous study (Dao et al., 2010) were probably due to the lower total MC content in MCCE. Specifically, MCCE of Dao et al. (2010) contained 6.92 mg L^{-1} of total MCs, whereas our MCCE contained 5.36 mg L^{-1} of total MCs derived from 670 µgMC g^{-1} dry weight of cyanobacterial bloom materials (see section 2.2). Therefore, higher beneficial compound concentrations, which are considered as the nutrients, were injected into the culture medium given with the specific concentrations (e.g. 50 µgMC L^{-1}). The bioactive compounds in CCEs included vital components, such as lipopeptides (40%), amino acids (5.6%), fatty acids (4.2%), macrolides (4.2%), and amides (9%) (Lau et al., 2015), which were likely to improve the *D. magna* fitness and, probably, the reproduction (Hulot et al., 2012). The possible effects of CCEs were definitely dependent on species-specific cyanobacteria (Hulot et al., 2012). Our study yielded interesting results showing that the reproduction-stimulated effects of two CCEs from two kinds of cyanobacteria species on *D. magna* were close to each other, possibly implying that either similar properties of two CCEs existed or at least some compounds that stimulated the reproduction were contained simultaneously in both CCEs (Hulot et al., 2012). Our results were in line with Dao et al. (2010) who observed the death of eggs and neonates, and neonate malformations of *D. magna* females exposed to 5 and 50 µgMC L^{-1} of MCCE or purified MC. Interestingly, in our study, the harmed offspring of MCCE and NCCE exposed *D. magna* were observed and this implies that not only the MC is responsible for the harmed offspring but also other unknown harmful compounds should be considered. In fact, Bednarska and Slusarczyk (2013) also proved that the microcystin-free filamentous cyanobacteria could cause the maternal abortion of *Daphnia pulicaria*. It is likely that the offspring of MCCE and NCCE exposed *D. magna* suffered from toxic pressures and therefore needed to pay the energy cost for resisting the toxins, even from the embryonic development stage (Dao et al., 2010). In addition, the degradation of offspring was also derived

directly from maternal biotransformation of MC and/or harmfully bioactive compounds to eggs (Wiegand, 2009). According to our knowledge, the modelizations of stimulated responses of life-history trait and physiological endpoints versus CCE concentrations have not been currently implemented. A few meta-analyses (e.g. Lurling, 2003; Herrera et al., 2015) indicated that the linear and logistic models were usually applied for describing correlations of adverse responses versus CCE concentrations or cyanobacterial cells. In this study, we suggested the quadratic equation, which is able to demonstrate the correlation between *D. magna* responses and CCE concentrations. Specifically, CCEs at low concentrations assumed under "a tolerance threshold" did support *D. magna* reproduction (e.g. the number of neonates per female) as the nutrient supplement, whereas as increasing CCE concentrations over such a tolerance threshold, harmful expressions were observed (Herrera et al., 2015). Hence, we suggest that further research with higher CCE concentrations should be performed to test the aforementioned hypothesis. Interestingly, among many endpoints belonging to the life-history trait and physiological responses evaluated, the number of neonates per female endpoint exhibited the highest correlation to both MCCE and NCCE exposures. It is reasonable as Cui et al. (2016) and Sancho et al. (2016) revealed that the number of neonates per female was determined as a very important endpoint for the ecotoxicological evaluation because it highly reflected the toxic levels of tested substances on model organisms.

The enhanced somatic growth of *D. magna* exposed to MCCE in our study was in line with Dao et al. (2010), of which *D. magna* was exposed to MCCE at concentrations of 5 and 50 μgMC L^{-1}. However, purified MC at concentrations of 5 and 50 μg L^{-1} also increased *D. magna* somatic growth (Dao et al., 2010). Therefore, our results confirmed that MC is likely to enhance the *D. magna* somatic growth and, beyond that, it is reasonable to consider other compounds responsible for the increase in somatic growth of CCE-exposed *D. magna*. Another highly possible scenario is the role of additional nutrients for stimulating the somatic growth which has been proven by Herrera et al. (2015). Specifically, the body length of *Moina micrura* and *Daphnia similis* was increased significantly relative to the control when exposed to MCCE at the concentrations of 4.5–21.7 μgMC L^{-1} (Herrera et al., 2015).

In our results, the stimulated feeding rate only obtained in MCCE-exposed *D. magna* but not in NCCE-exposed *D. magna*. Interestingly, according to Ghadouani et al. (2004), exposure to purified MC at a concentration of 50 μg L^{-1} did not change the feeding behaviors of *Daphnia pulicaria*. In addition, exposure to MCCE at a concentration of 134.5 μgMC L^{-1} strongly inhibited the *Daphnia similis* feeding behaviors (Herrera et al., 2014). Thus, in our study, there must be some of the unknown substances at the relevant concentrations contained in our investigated MCCE. We suggest the chemical composition in the two CCEs should be further determined to clearly interpret differences in the feeding rate effects of both CCEs.

3.5. Conclusion

In summary, *D. magna* survival rate was insignificantly reduced by both CCEs. Besides, the fertility of *D. magna* exposed to both CCEs was drastically improved, even at the lowest concentration. Although the reproduction-stimulated effects on *D. magna* expressed obviously at evaluated endpoints, the offspring-toxic effects were also recorded in all MCCE and NCCE exposures. Besides,

D. magna somatic growth was also increased under exposures to MCCE and NCCE at all the tested concentrations. Interestingly, the feeding rate was only enhanced by MCCE exposures, which indicated that there were some of the dissimilar properties between CCEs that remained unknown. Here, our results were new and interesting that ecotoxicologically indicate the feeding rate as well as life-history trait responses of *D. magna* to MCCE and NCCE at its early stage of development. The toxicities of *Pseudanabaena* sp. extract on *D. magna* obtained in our study were considered as the first report for further investigations. Ecotoxicological tests with reservoir water collected directly from where cyanobacterial blooms occurred by using *D. magna* and/or the variety of species will be investigated in the future.

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4. DIFFERENT RESPONSES OF *SIMOCEPHALUS VETULUS* (CLADOCERA: DAPHNIDAE) TO FIVE ANTIBIOTICS AND THEIR MIXTURE UNDER 48-HOUR ACUTE TEST

4.1. Introduction

Antibiotic use is increasing globally, particularly in low- and middle-income nations (Klein et al., 2018). In addition, the current global-scale increase of antibiotic consumption has been from 26.2% in Access group antibiotics and up to 90.9% in Watch group antibiotics; namely, Watch group antibiotic use tends to increase significantly (Roberts and Zembower, 2021). Therefore, it's no surprise that antibiotic pollution has a significant impact on the environment, economy, and society, resulting in ecosystem degradation and the spread of antibiotic-resistant microorganisms (Kraemer et al., 2019; Amarasiri et al., 2020).

Fluoroquinolones and tetracyclines are two of the fastest-growing antibiotic groups (Schar et al., 2020; Tomas et al., 2021). Hence, more than 90% and 75% of eaten fluoroquinolones and tetracyclines, respectively, are released into the environment in the active form (Janecko et al., 2016; Xu et al., 2021). In addition, fluoroquinolones are synthesized antibiotics and thus showed the persistence to biological degradation (Sarangapani et al., 2019). Due to its high hydrophilicity and low volatility, tetracyclines are challenging to treat efficiently in conventional wastewater treatment facilities, and wastewater from pharmaceutical zones and hospitals contains high concentrations of fluoroquinolone and tetracycline (Daghrir and Drogui, 2013; Booth et al., 2020). As a result, more attention should be directed to fluoroquinolones and tetracyclines. It's worth noting that they've been proved to harm eukaryotic organisms both directly and indirectly (Moullan et al., 2015; Nunes et al., 2018; Lu et al., 2019; Akbar et al., 2020; Motiei et al., 2020), even though they are principally designed to have an affinity with prokaryotic cells (Xie et al., 2010).

In the fluoroquinolone group, the second-generation antibiotics ciprofloxacin (CFX) and ofloxacin (OFX) are two of the most consumed antibiotics in the world (Hsia et al., 2019; Xu et al., 2020). CFX and OFX residues reaching 31,000 μg L^{-1} (Larsson et al., 2007) and 3,330 μg L^{-1} (Hussain et al., 2016), respectively, were detected in effluent from the wastewater treatment plant of drug manufacturers. On the other hand, the third-generation antibiotic gatifloxacin (GFX) is applied for ophthalmic treatment and is banned from using as an oral dosage due to serious adverse side effects (Schultz, 2012). Antibiotics whose targets can be discovered in eukaryotic cells have a potential affinity for eukaryotic cells, which is widely established (Barnhill et al., 2012; Zareifopoulos and Panayiotakopoulos, 2017; Fief et al., 2019). In fact, that 3.7 μg L^{-1} of GFX residue has been detected in the municipal wastewater treatment plant (Mutiyar and Mittal, 2014). Moreover, delafloxacin (DFX), a newly approved antibiotic of fourth-generation fluoroquinolone, was released in 2017. The advantage of DFX is that it is highly effective against classical fluoroquinolone-resistant pathogens; it also performs well in acidic environments when the activity of other fluoroquinolones is significantly diminished (Candel and Peñuelas, 2017). So, high expectations of increasing consumption of DFX in therapy are reasonable. DFX residues as a water pollutant, on the other hand, have gotten little attention because the efficacy and safety of DFX are still being researched before it is extensively used in practical treatment (Lan et al., 2019). DFX, on the other hand, is an anionic antibiotic that is thought to be ineffectively removed by traditional wastewater treatment facilities

(Liu et al., 2018). In addition, Dang et al. (2021) showed that DFX is less adsorbed on biochars, which are expected to be applied to post-treatment systems in wastewater treatment plants. As a result, it is reasonable to concern that DFX is perhaps soon detected in various water environments. On the other hand, tetracycline (TET) is well known to have numerous advantages, such as few side effects (Inta et al., 2017) and low cost in the therapy (Jahantigh et al., 2020); therefore, TET is the most broadly used antibiotic for both humans and animals since many decades (Kang et al., 2018). For example, TET is currently one of the most used antibiotics for poultry farms (Di Francesco et al., 2021). Then, TET residues are frequently detected with high concentrations, such as up to 2,600 μg L^{-1} in effluent from the wastewater treatment plant of drug manufacturers (Hou et al., 2016).

The toxic levels of fluoroquinolones and TET have been clarified according to the 48-hour standard acute immobilization tests using *Daphnia magna* based on OECD protocol (OECD, 2004). Specifically, the most toxic values of CFX (Dionísio et al., 2020), OFX (Isidori et al., 2005), and TET (Havelkova et al., 2016) were reported to be 36.49, 31.75, and 8.16 mg L^{-1} as 48-hour $EC_{50,}$ respectively. However, other studies showed higher 48-hour EC_{50} values for CFX (Załęska-Radziwiłł et al., 2011; Martins et al., 2012; Dalla Bona et al., 2014) and TET (Wollenberger et al., 2000; Sönmez and Sivri, 2020). However, toxic levels obtained from 48-hour acute tests of CFX, OFX, and TET were varied for the different model organisms (Isidori et al., 2005; Dalla Bona et al., 2014; Havelkova et al., 2016). It is noteworthy that non-standard acute biomarker tests using various model organisms for CFX, OFX, and TET are highly sensitive regardless of tested model organisms (Peltzer et al., 2017; Yang et al., 2019; Dionísio et al., 2020; Nogueira and Nunes, 2020). Biomarkers typically consist of antioxidant enzymes, such as glutathione-S-transferase and catalase (Peltzer et al., 2017; Dionísio et al., 2020; Nogueira and Nunes, 2020). It should be highlighted, however, that biomarkers are employed as a potential tool for explaining toxic mechanisms, not as a baseline for measuring hazardous levels, because negative effects on biomarkers do not always lead to negative impacts on phenotypes of tested animals, such as behavior (Nogueira and Nunes, 2020), morphology (Peltzer et al., 2017), and life-history traits (Nunes et al., 2018). As shown here, the toxic level can widely vary depending on different model organisms and endpoints. This is an unavoidable issue in the theory of ecotoxicological testing. As a result, when comparing hazardous levels among different toxicants is of relevance, toxicity studies must be constructed using the same organisms and endpoints (Verma, 2008).

Currently, only two studies are found on the toxicity test of GFX. Mala and Dutta (2019) reported that a toxic level of GFX to *D. magna* immobilization was 48-hour EC_{50} of 330.8 mg L^{-1}. Shen et al. (2019) investigated the acute response of zebrafish for morphological abnormalities, physiology, and gene-expressed. They showed a significant effect of GFX at 3.713 mg L^{-1}. DFX, on the other hand, has yet to be subjected to an ecotoxicological test. Given the possible concerns these antibiotics provide to the aquatic environment, comparative studies of antibiotics should be carefully considered. In this comparison, of course, the selection of endpoints and model organisms is very significant to assess the toxic levels. In addition, considering the principle objectives of the ecotoxicological study, the toxicity test should be performed at endpoints related to the life history of the organism (Rudén et al., 2017). Of course, it is imperative to test the effects on reproduction and development because such effects are directly related to changes in proliferative capacity in the environment (Ananthasubramaniam et al., 2015), and they can be easily observed, such as the morphological abnormalities of the organism and the number of offspring (Xiang et al., 2011; Grzesiuk et al., 2020).

On the other hand, more focus needs to be placed on the effects of *toxicants* (e.g., antibiotics) on the behavioral and physiological properties of living organisms because they can change the fitness of organisms in the ecosystem even if toxicants do not directly affect mortality or immobilization (Tkaczyk et al., 2021).

Because this endpoint is directly correlated with the capture of food particles such as phytoplankton cells or detritus, and oxygen supply as a result of the continuous flow of water, thoracic limb activity as a physiological property of the water flea is a vital endpoint to evaluate model organism response to toxicants (Tkaczyk et al., 2021). In addition, thoracic limb activity is also closely associated with immobility and death; therefore, this endpoint can be used as an assessment tool in acute toxicity tests (U.S. EPA, 1994). Moreover, as mentioned, the 48-hour standard acute immobilization tests commonly require very high concentrations of exposure to obtain immobility (or even death). Therefore, in the case of tested expensive medicines, the cost of the experiment is also an issue. Therefore, in evaluations of pharmaceutical effects on the model organisms, thoracic limb activity is among the highly recommended endpoints because results can be obtained with lower concentrations and shorter exposures (Tkaczyk et al., 2021). However, the current report of ecotoxicological tests for antibiotics using this endpoint is minimal (Bownik et al., 2019; Tkaczyk et al., 2021). Appreciably, the thoracic limb activity of model organisms under exposure to fluoroquinolones and tetracycline are not available.

In this test, we selected *Simocephalus vetulus* as a model organism. One of the water fleas has been used as a model organism in ecotoxicological tests (Arias et al., 2020); however, it is not a standard model organism that is assigned in the toxicity testing protocols (U.S. EPA, 2002; OECD, 2004; ASTM, 2014). Because *D. magna* and *S. vetulus* are both members of the Daphniidae family, they share life cycle characteristics. Although *S. vetulus* lifespan is considerably shorter than that of *D. magna* (Wu et al., 2007). *S. vetulus* can be found worldwide in freshwater systems of continents, unlike *D. magna,* which is only found in temperate regions (Tsui and Wang, 2007, Wu et al., 2007; Kohout et al., 2014; Etilé et al., 2020). As a result, it is a very vulnerable species in freshwater habitats when it comes to contaminants like antibiotics (Wu et al., 2007). Therefore, it is considered that *S. vetulus* is a suitable model organism that can be used worldwide for the ecotoxicological test. In this study, we investigated *S. vetulus* thoracic limb activity under the 48-hour acute exposure to fluoroquinolones, tetracycline, and their mixture to infer the different toxicological behaviors of those antibiotics in single and combined forms.

4.2. Materials and methods

4.2.1. Test chemicals and stock preparation

The powders of CFX (CAS number: 85721-33-1; 98% purity) and GFX (CAS number: 180200-66-2, 98% purity) supplied by LKT Laboratories Co., Ltd., OFX (CAS number: 82419-36-1, 98% purity) supplied by Fujifilm Wako Pure Chemical Industries, Ltd., DFX (CAS number: 189279-58-1, 98% purity) supplied by Sigma-Aldrich Co. LLC., and TET (CAS number: 2058-46-0, 95% purity) supplied by Nacalai Tesque, Inc., were used for the tests. Molecular structures, molecular weights, and physical properties of those antibiotics were exhibited in Fig. A-1. Stock solutions of each antibiotic were prepared separately in pure water (Elga® PURELAB® Water Purification System,

United Kingdom) at the final concentration of 0.25 g L^{-1}. Acidic condition (pH 2–3) was essential for totally dissolving CFX, OFX, and GFX; therefore, a few drops of 1 M HCl were added to the stock solution of 100 mL. Contrarily, alkaline condition (pH 10–11) was favorable for dissolving DFX. Thus a few drops of 1 M NaOH was added to the stock solution of 100 mL. For TET, the stock solution of 100 mL was prepared directly by pure water. After that, 1 M HCl or 1 M NaOH was added to neutralize the stock solution. All stock solutions were stored at 4 ºC in the dark during the use.

4.2.2. Clone collection of test organism and culture maintenance

A population of cladoceran *S. vetulus* was collected in a pond in Nagasaki University, Japan (GPS coordinates: 32.7851, 129.8646). The collected population of *S. vetulus* was transferred to a laboratory and was nourished individually in 50 mL flasks containing 50 mL medium of this pond in 16 h light (700–800 lux) /8 h dark and the temperature of 20 ºC in the incubator (EYELA Co., Japan). We started with 10 organisms without eggs in the brood chamber. The culture medium was changed every other day by this pond water. Until offsprings in those flasks were released, we replaced pond water with dechlorinated tap water, and organisms were fed with one droplet (approximately 20 μL) of the mixed food consisting of yeast powder (Nissin, Japan) and a powder food for fry (Sera Micron, Germany) (food stock contained 4.5–5.0 g L^{-1} of total solids, adjusted from Environment Canada, 2007). Tracking the neonates in each flask during three more times of medium exchange (i.e., 6 days), we then selected a flask with the healthiest organisms expressed by fast growth and dark-color body (see Fig. A-2). For long-term culture maintenance, all organisms in a chosen flask were transferred to a 1-liter beaker containing 1 liter dechlorinated tap water. Every other day, we added 1 mL of the mixed food and changed the culture medium. Before the formal ecotoxicological test, the organisms were acclimated for a year.

4.2.3. Acute ecotoxicological test design

Prior to the ecotoxicological tests, a population of neonate *S. vetulus* less than 24 hours old was isolated and cultured until each individual became the gravid female. As shown in Fig. 3, the progeny of those gravid females was monitored according to birth sequence and employed for the ecotoxicological test. This work is to eliminate the potential effects of the maternal birth sequence (or maternal age) on the intrinsic thoracic limb activity, as *S. vetulus* lifespan is relatively short that perhaps results in significant differences in neonate properties among births (Nandini and Sarma, 2000; Šorf et al., 2017).

Before the test 5 hours, less than 24 hours old neonates were isolated, transferred to a 100 mL beaker filled with 100 mL dechlorinate tap water, and fed the mixed food of 0.1 mL.

The experimental design was based on the acute test procedure of Cui et al. (2018). Eight nominal concentrations of 0, 0.625, 1.25, 2.5, 5, 10, 20, and 40 mg L^{-1} for each antibiotic and their mixture (MIX) were prepared to dissolve to dechlorinate tap water. These exposure concentrations are expected not to cause the lethal effect to *S. vetulus* concerning 48-hour EC_{50}(s) of CFX, OFX, GFX, and TET for the immobilization responses of a standard model organism *D. magna* in the previous studies (Wollenberger et al., 2000; Isidori et al., 2005; Załęska-Radziwiłł et al., 2011; Martins et al.,

2012; Dalla Bona et al., 2014; Mala and Dutta, 2019; Sönmez and Sivri, 2020). Table A-2 shows the molar concentration of each antibiotic in each exposure concentration of MIX. Since we aimed to clarify the non-additive effect of the antibiotic cocktail on *S. vetulus* thoracic limb activity, the molar-based concentration fractions are equivalent to the fractions among the antibiotic concentration of the single exposure (Zhang et al., 2021).

Ten neonates were distributed individually to ten wells in a 24-well culture plate filled with 3 mL of antibiotic-containing medium or antibiotic-free medium (control). Namely, 10 replicates were tested for each exposure condition. Then, the culture plates were placed in the same incubator (20 ºC) used for the maintenance culture with the dark condition and no feeding during 48 hours in the test (OECD, 2004). The degradations of DFX and GFX were only measured representatively for the lowest and highest concentrations (i.e., 0.625 and 40 mg L^{-1}) at the start of exposure and after the ecotoxicological tests (48 hours later), by using HPLC (JASCO Co., Ltd., Japan). This analytical method followed our previous work (Dang et al., 2021). The pH (7.65–7.90) and hardness (65–70 mg L^{-1} as $CaCO_3$) of the medium were also measured at the start and 48th hour of tests. The mortality and thoracic limb activity were only evaluated at the 48th hour to prevent causing physical discomfort to the infants and disrupting their ocular condition.

4.2.4. Measurement of thoracic limb rate

After 48 hours from the start of the exposure, each individual of *S. vetulus* was gently transferred from the culture plate to the zooplanktonic counting plate (RIGOSHYA, Co., Ltd., Japan) by a plastic dropper. After 10 seconds for acclimatization, subsequently, the thoracic limb activity of each individual was recorded for 6 seconds using a microscope (Eclipse 50i, Nikon Co., Ltd., Japan) equipped with a digital camera (EOS 60D, Canon Co., Ltd., Japan). *S. vetulus* has the characteristic of showing very little movement, unlike the jumping-like movement type of *D. magna* (Jiang et al., 2016). Therefore, it was unnecessary to use additional chemicals such as glue to fix the individual (Pan et al., 2017).

The videos of thoracic limb activity of individuals were analyzed by Adobe Premiere Pro (Adobe Inc., USA). Thoracic limb rate (TLR) was defined as the number of thoracic limb beats in 6 seconds. The video was played one-tenth slower than normal to count thoracic limb beats. The mortality of *S. vetulus* was also counted during this TLR measurement. If no thoracic limb activity was recorded during the recording period, the individual's death was determined by gently touching the person three times with the plastic dropper.

4.2.5. Data analysis

R software (version 4.0.4, R: A language and environment for statistical computing, Foundation for Statistical Computing, Vienna, Austria, URL https://www.R-project.org/.) was used on RStudio (version 1.4.1106, Lucent Technologies, Inc., USA) for all data analysis. The type I error level was set at a p-value < 0.05, which was considered a statistical criterion to reject the null hypothesis of each test.

Counted events such as thoracic limb beats in a time interval show Poisson distribution (Plan, 2014). However, since the enumerated number of thoracic limb beats of an individual determined for each test ranged between 20.7–34.2, it was judged that the normal distribution approximation for the random variable could be applied (Siegel, 2011). Therefore, the one-way ANOVA was used, followed by Tukey's HSD test ("multcomp" package) to detect the significant differences between controls among tests or Dunnett's test ("multcomp" package) to detect the significant differences between the antibiotic exposures (in each antibiotic test) and the corresponding control. The normality of residuals and homogeneity of variances were confirmed by the Shapiro-Wilk test and Levene's test ("car" package), respectively. In cases of violated assumptions for homogeneity of variances, Welch's ANOVA followed by multiple independent *t*-tests (unpooled variances) with *p*-values adjusted by Benjamini and Hochberg method was applied. Otherwise, the Kruskal-Wallis H test followed by many-to-one Gao's test (Gao et al., 2008) ("nparcomp" package) with *p*-values adjusted by Benjamini and Hochberg method, was used as an ultimate alternative.

4.3. Results and discussion

4.3.1. The degradation of antibiotics and the antibiotic effects on the survival in the exposure period

Table 4-1 shows the initial and the terminal concentrations of DFX and GFX. The measured concentration of DFX was lower than the nominal concentration, whereas the concentration of GFX was higher than the nominal concentration. However, since the errors were less than 13%, and the results indicated no degradation of the two antibiotics during 48 hours of tests. In addition, previous studies also resulted in no significant degradation of CFX and OFX within 48 hours (Kümmerer et al., 2000; Dalla Bona et al., 2015), whereas approximately 9.7% TET degradation was detected after 48 hours (Xie et al., 2019), where experimental conditions were the same as this study. Therefore we judged that the five antibiotics in this study remained within ±20% of the nominal concentrations during 48 hours in each test; the results were interpreted as though they were obtained with nominal quantities of exposure (Dalla Bona et al., 2015).

No mortality was detected in exposures to DFX, TET, CFX, OFX, and MIX as well as the controls, whereas the mortality of 90% and 100% were found in GFX exposures of 20 and 40 mg $L^{-1,}$ respectively. Thus, we excluded GFX exposures of 20 and 40 mg L^{-1} from TLR analysis. Mala and Dutta (2019) found that even at 1000 mg L^{-1}, GFX only caused 65% mortality for neonate *D. magna* after 48h. Thus, *S. vetulus* is thought to have a high sensitivity for ecotoxicological testing. In fact, earlier research has revealed that S. vetulus is more sensitive to chemical substances than *D. magna* (Hickey, 1989; Santos-Medrano and Rico-Martínez, 2019).

Table 4-1. Analytical results for delafloxacin (DFX) and gatifloxacin (GFX) in the initial and terminal medium.

Antibiotic	Nominal concentration (mg L^{-1})	Initial concentration (mg L^{-1}) (n = 3)	Terminal concentration (2 days later) (mg L^{-1}) (n = 3)
DFX			
	0.625	0.583 ± 0.018	0.583 ± 0.087
	40	38.17 ± 2.49	35.10 ± 2.65
GFX			
	0.625	0.702 ± 0.023	0.698 ± 0.007
	40	41.79 ± 0.14	40.6 ± 0.46

4.3.2. The difference in thoracic limb rate of different birth neonates

Figure 4-1 shows a series of captured images from a video about the pulsation of the thoracic limb activity. The side views of A-E are a sequence of photos taken during one period of thoracic limb vibration motion, with each green line on the image representing the fringe of the moving thoracic limb. The red arrow shows the direction of motion. F-set J's of photos also demonstrates motion in front perspectives. We could precisely count the thoracic limb's beats for six seconds to determine each individual's TLR.

In our study, ecotoxicological tests with DFX, GFX, TET, CPX, and OFX and MIX were performed by using the neonates in births 2–6, respectively. Fig. 4-2 showed the change of intrinsic TLRs of neonates in the controls from births 2–6. One-way ANOVA proved that the measured TLRs at each birth was not equal ($p < 0.001$, Table A-3). Moreover, each asterisk mark in Fig. 2 indicated the significant level of the multiple comparisons (by Tukey's HSD test). This statistical analysis evidenced the decrease of TLRs from births 2–4 and the slight increase at birth 6.

In this experiment, the maternal life span of *S. vetulus* for the ecotoxicological test was approximately 54 days and had around 11 births under our nourishment, which was consistent with the previous studies (Nandini and Sarma, 2000; Šorf et al., 2017). Therefore, the maternal ages of *S. vetulus* could modulate offspring properties rapidly via the trade-off mechanism with the brood size (Nandini and Sarma, 2000; Guinnee et al., 2007) and/or maternal fitness (Boersma, 1997; Bock et al., 2019). Although *S. vetulus* has been utilized for ecotoxicological experiments, a particular guideline for

ecotoxicological tests utilizing S. vetulus as a model organism has not yet been developed to deal with this aging impact (Hickey, 1989; Wu et al., 2007; Santos-Medrano and Rico-Martínez, 2019; Arias et al., 2020). In this instance, we propose that an experimental design with proper allocation for each birth be used to statistically exclude the influence of mother age (or birth/time) when comparing multiple antibiotic exposures. However, since this study was the first experiment to test the effects of antibiotics on TLRs using *S. vetulus*, the effects of antibiotic exposures were determined by internal comparisons to the corresponding control to clarify the alternation of TLRs induced by antibiotic exposures sharply.

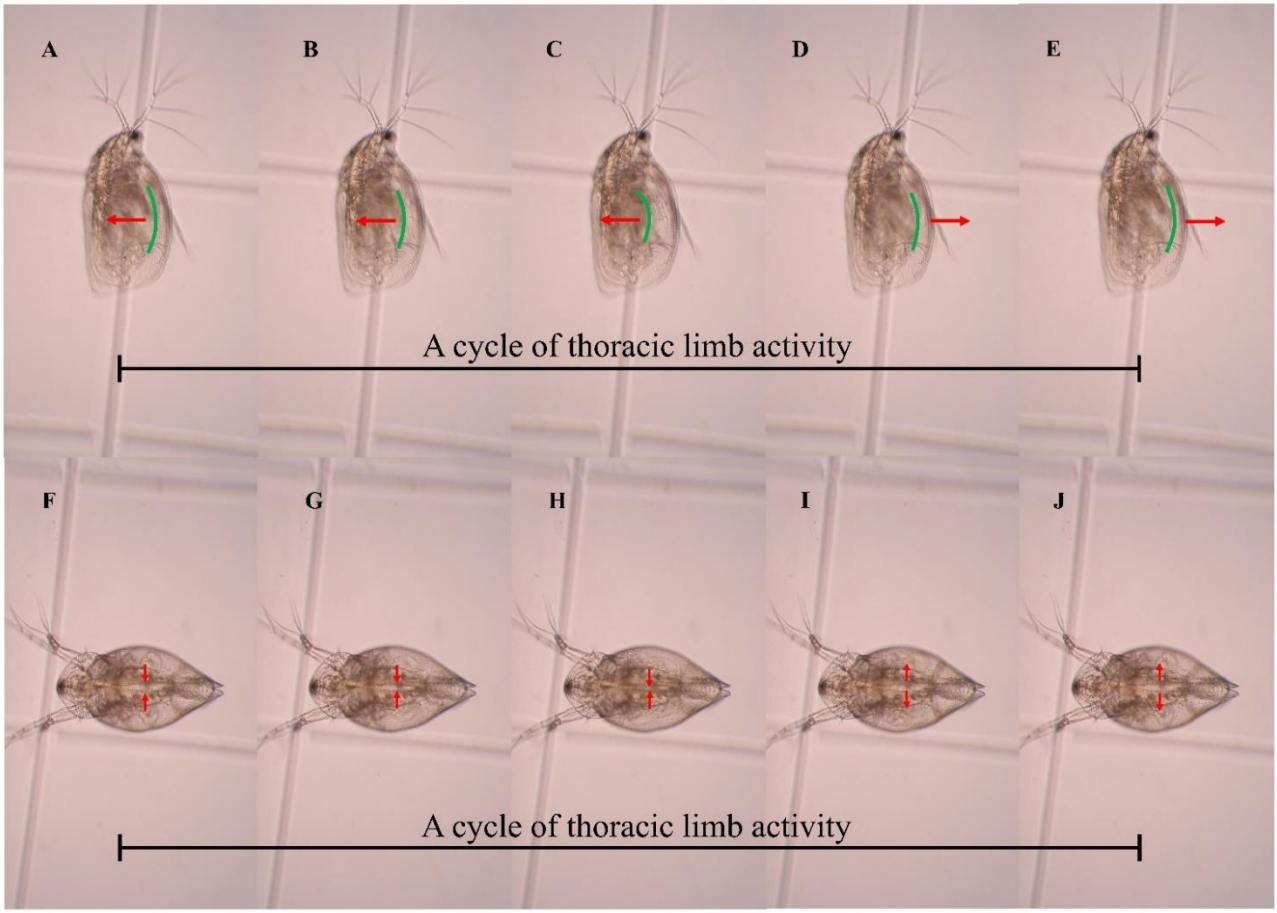

Fig. 4-1. Illustrations of the cycle determination of thoracic limb activity. Photos A-E indicate the side-view type. Photos F-J indicate the bottom-view type. Green arcs indicate the moving thoracic limb and red arrows indicate the moving direction.

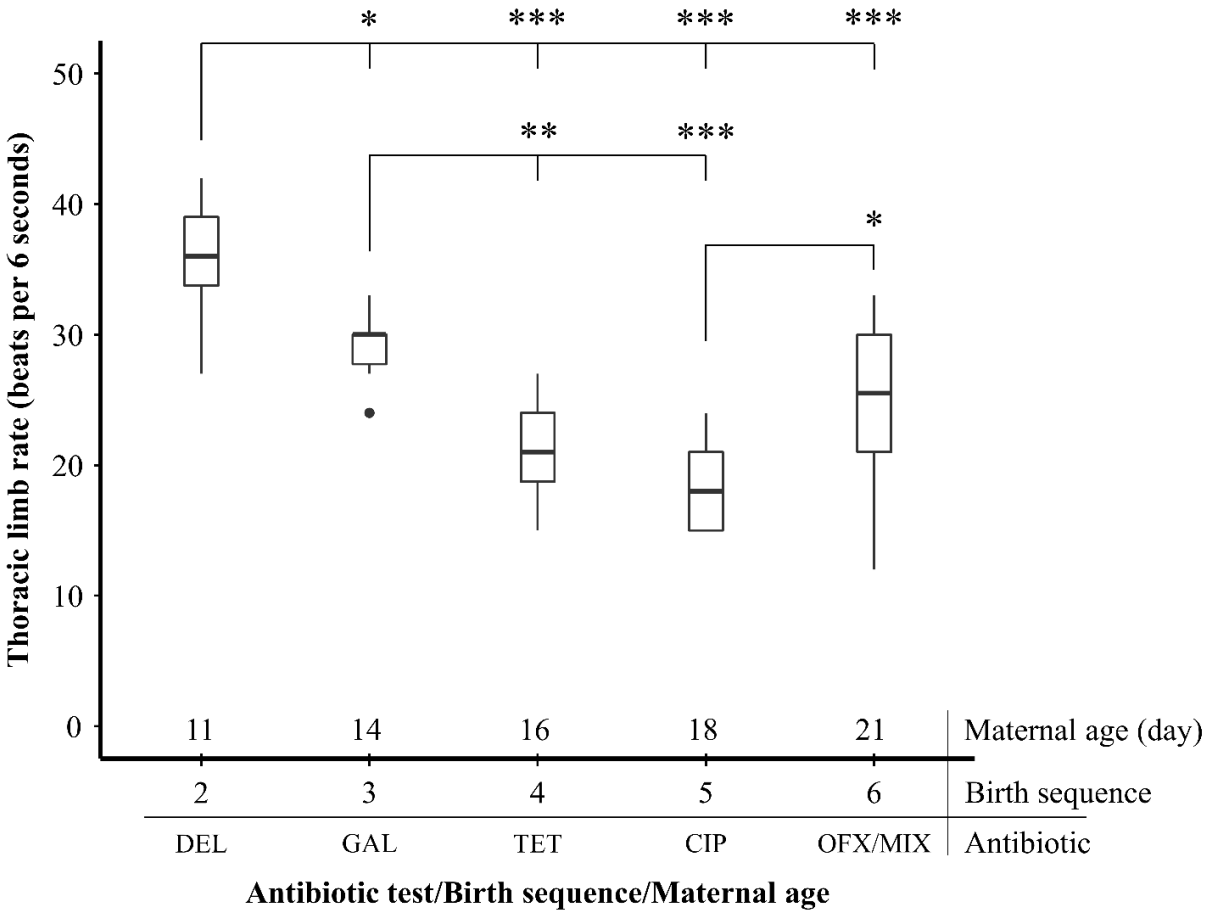

Fig. 4-2. Box and whisker plot of intrinsic thoracic limb rate of control *Simocephalus vetulus* in five single antibiotic tests (CFX–ciprofloxacin, DFX–delafloxacin, GFX–gatifloxacin, OFX–ofloxacin, and TET–tetracycline) and an antibiotic cocktail test (MIX–mixture of five antibiotics) with respect to birth sequence and organism age. Asterisks indicate the levels of statistically significant differences: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

4.3.3. The effects of the five antibiotics and antibiotic cocktail on thoracic limb rate of *S. vetulus*

Fig. 4-3 showed results of TLRs measurement in all exposure conditions. Statistical comparisons are possible only under the same antibiotic exposure conditions with neonates of the same birth, as mentioned in the previous section. However, clear trends can be arguable between exposures to each antibiotic. Specifically, Table A-3 and Fig. 4-3 clearly show three types of responses: the first is no significant effect, the second is the reduction of TLRs, and the third is the activation of TLRs. Multiple comparison tests have shown that statistically significant suppression of TLRs occurred in the GFX exposure at 2.5 mg L^{-1} and higher concentrations of GFX and in 40 mg L^{-1} in the MIX exposure. Given the high mortality in GFX exposures, this low concentration causing the inhibitory effect is convincing. In addition, the MIX contains approximately 21% GFX (see Table S1), suggesting that TLR suppression occurred. Conversely, in CFX exposure, significant increases in TLRs occurred onward at 10 mg L^{-1} and 1.25 mg L^{-1} for OFX exposure. On the other hand, the

results of ANOVA tests show that there was no significant effect of DFX and TET exposure on TLRs of *S.vetulus* (*p*-values > 0.05, Table A-3).

Unlike many other fluoroquinolones, such as CFX, OFX, and GFX, which are designed to have high antibacterial activity in neutral settings while reducing antibacterial activity in acidic situations, GFX was designed to have high antibacterial activity in both neutral and acidic conditions (Tulkens et al., 2019). The absence of the protonable substituent in position 7 (see Fig. A-1) is an essential design to allow DFX to be neutral at acidic pH and anionic form at neutral pH (Van Bambeke, 2015). In an anionic form, DFX has low membrane permeability across cells and low absorption on the organism's surface and intestinal system compared to zwitterionic and neutral forms (Völgyi et al., 2012). Similarly, the reason for the no effect on TET exposure may be related to pH conditions. TET is most active at pH 6.0–7.0, where the high capacity of membrane permeability was expressed due to the predominance of TET zwitterion form (Zhang et al., 2014); our testing settings are kept at a pH range of 7.65–7.90. As a result, the lack of a substantial TET result could be due to a lack of active form. TET is also known to have lower anti-eukaryotic action than fluoroquinolones. For instance, TET cannot act to the highly complex processing of eukaryotic rRNA (Chopra and Roberts, 2001; Chukwudi, 2016). For these reasons, DFX and TET could not affect to TLRs of *S.vetulus*.

GFX, on the other hand, is perhaps to be a strong pro-oxidant in the fluoroquinolone group (Park-Wyllie et al., 2006; Nunes et al., 2018). Furthermore, the tautomer ratio (the concentration ratio of the zwitterions to neutral species) is remarkably higher than other fluoroquinolone antibiotics, including CFX and OFX, implying that GFX has a higher capacity for membrane permeability (Völgyi et al., 2012). Therefore, GFX intracellular concentration can be high enough to cause oxidative stress and, eventually, deaths of cells and individuals.

CFX and OFX caused an increase in TLRs, as shown in Table A-3 and Fig. 4-3. The multiple comparison test of CFX exposures to the control revealed significant increases in TLR at 10–40 mg L^{-1} and OFX exposures significantly increased TLRs at 1.25–40 mg L^{-1}. In both CFX and OFX exposures, TLRs had the trend to be decreased at 40 mg L^{-1} compared to at 20 mg L^{-1}. Calabrese and Baldwin (2003) mentioned the inverted U shape response of endpoints to the different concentration exposures of antibiotics. This U shape response relates to a hormesis effect. The hormesis effect is generally recognized as a positive or beneficial response of organisms in exposures to a toxicant of low or moderate concentration below the tolerance threshold (Calabrese and Mattson, 2017). This U shape pattern is seen in the CFX and OFX exposures in Fig. 4-3. Fluoroquinolones CFX and OFX produced oxidative damage in exposed species, as previously stated (Nunes et al., 2018). Antibiotic invasion is thought to produce reactive oxygen species (ROS) in cells, which can cause oxidative stress in organisms (Zhang, 2018). The production of reactive oxygen species (ROS) in cells is proportional to oxygen consumption (Borgeraas and Hessen, 2000). Aderemi et al. (2018) has already elucidated that CFX causes the increase of ROS, followed by increasing oxygen consumption in microalgae *Raphidocelis subcapitata*. In other words, since higher antioxidant activities demand much oxygen to reduce ROS in cells (Bownik et al., 2019; Tkaczyk et al., 2021). Therefore, in our results, CFX and OFX exposures perhaps caused the increment of TLRs to supply much oxygen to the cell. On the other hand, OFX affected the TLR in a lower concentration than CFX. This result is likely to be due to the higher membrane permeability of OFX than CFX because of the higher

proportion of zwitterion and higher lipophilicity under the pH conditions of our tests (Völgyi et al., 2012).

Only the highest dosage of 40 mg L1 suppressed the TLR endpoint in the MIX exposure (Table A-3 and Fig. 4-3). If only GFX has a significant effect on *S. vetulus* in MIX exposure, a decrease in TLR should be found in the 20 mg L^{-1} of MIX exposure because MIX of 20 mg L^{-1} contains 10.66 μM of GFX (see Table A-2). In fact that this concentration is higher than the molar concentration of 2.5 mg L^{-1} of GFX exposure in which GFX could reduce the TLR (see Table A-21 and Fig. 4-3). The MIX solution contains approximately 21% of GFX, 23% of CFX, and 21 % of OFX as molar concentration fractions. Therefore, if CFX, OFX, and GFX cause similar oxidative stress with different intensities, it is possible to reduce TLRs at low concentrations of MIX exposure and to show hormesis effects at even lower concentrations. However, experimental results suggest that the effect of the antibiotic cocktail (MIX) is determined by a balance between the two opposite tendencies of TLR-enhanced effect (hormesis effects) and TLR-suppressed effect. Hence, the mechanism of action of GFX on S.vetulus TLR is likely to be distinct from that of OFX and CFX.

Several studies already found that the antibiotic mixture induced lower effects than single antibiotics (De Liguoro et al., 2009; Dalla Bona et al., 2014; Zhang et al., 2021). In addition, it is argued that antibiotics having the same mechanism of action will have a synergistic effect (Yilancioglu, 2019). The four fluoroquinolones in this mixture have the same potential mechanism of action on prokaryotes and eukaryotes. Fluoroquinolones bind with topoisomerase II and DNA to produce an inactive complex, which prevents replication (Elsea et al., 1992; Blondeau, 2004). However, this action is unlikely to affect TLRs by two days of exposure. The effect on TLRs is assumed to be caused by oxidative stress on eucaryotic cells as a secondary effect, as previously described. As a result, the direct interaction of the antibiotic with the antibiotic in the combination is a viable explanation for the experiment's results (Pai et al., 2006). Specifically, the presence of an antibiotic (or antibiotics) inhibits another antibiotic (or other antibiotics) from crossing the cell membrane due to causing the change of the cell metabolism; therefore, the total bioavailability of antibiotics is insufficient to cause damages (e.g., oxidative stress) (Yilancioglu, 2019). However, the primary mechanism for this problem is still unresolved due to the unpredictable occurrence of synergism and antagonism relationship among antibiotics in the mixture (Rodea-Palomares et al., 2015). Therefore, more research is needed to explain the surprising findings of our study.

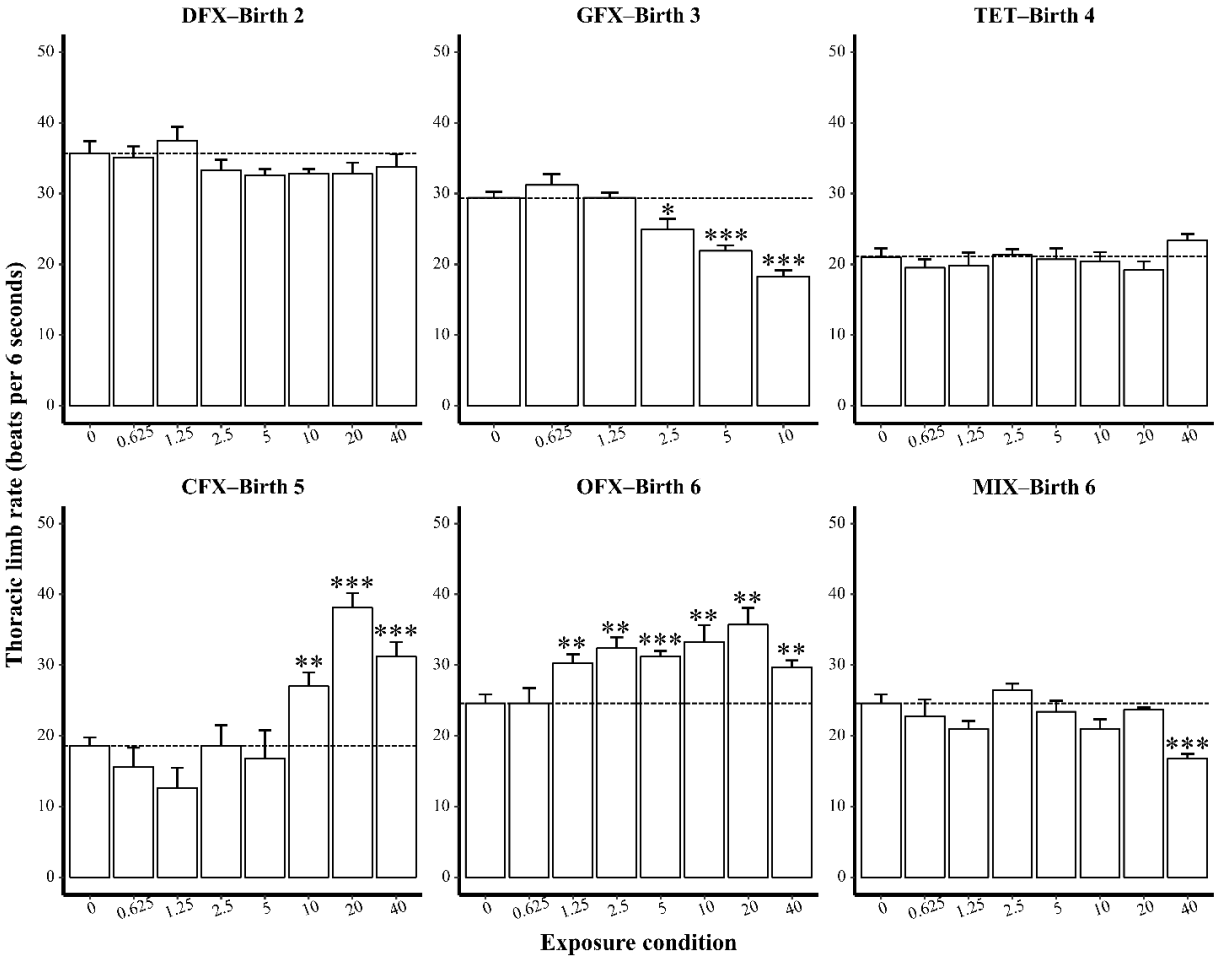

Fig. 4-3. The effects of exposure conditions on *Simocephalus vetulus* thoracic limb rate in five single antibiotic tests (CFX–ciprofloxacin, DFX–delafloxacin, GFX–gatifloxacin, OFX–ofloxacin, and TET–tetracycline) and an antibiotic cocktail test (MIX–mixture of five antibiotics). The bar graphs are expressed as mean ± standard error. Asterisks indicate the levels of statistically significant differences: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

4.4. Conclusion

Mortality Mortality of *S. vetulus* was nil in the tests of CFX, OFX, DFX, TET, and MIX after 48-hour exposures, except for GFX exposures of 20 and 40 mg L^{-1}. For ecotoxicological tests for single antibiotic and antibiotic mixture were performed using neonates at different births (births 2–6), and TLRs were affected by birth order; the effects of each exposure of the five antibiotics and MIX were statistically analyzed by comparing to corresponding controls. Due to the strong toxicity of GFX, a significant reduction in TLR of *S. vetulus* was observed at 2.5–10 mg L^{-1} GFX exposures. On the other hand, we found an increase of the TLRs when exposing the organisms to 10–40 mg L^{-1} for CFX and to 1.25–40 mg L^{-1} for OFX. The increases of TLRs in OFX and CFX exposures were interpreted to be a sort of hormesis effect. These effects on TLRs were considered to be caused by the oxidative stress of these antibiotics to eucaryote cells. However, DFX and TET exposures showed no significant effects on the TLRs. The low membrane permeability of DFX and TET under the pH conditions of the experiment could be the reason for no effect on TLRs. On the other hand, it was

previously considered that GFX's high toxicity was linked to its high membrane permeability. TLRs were expected to be lowered at 20 mg L1 because to the molar concentration ratio of antibiotics in MIX exposures, and exposure to the lower concentration was expected to have the hormesis effect. The exposure to MIX, on the other hand, only resulted in a reduction in TLR at 40 mg L1. The interaction of the antibiotics in the cocktail was thought to be the cause of this finding. From these results, we judged that TLR endpoint in an ecotoxicological test using *S. vetulus* is a useful tool for fast screening investigations of toxic levels of toxicants.

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5. CHRONIC ECOTOXICOLOGY AND STATISTICAL INVESTIGATION OF CIPROFLOXACIN AND OFLOXACIN TO *DAPHNIA MAGNA* UNDER EXTENDEDLY LONG-TERM EXPOSURE

5.1. Introduction

Antibiotics currently hold irreplaceable roles in disease treatment for humans and animals (Allen et al., 2013). Besides, aquaculture and land-based agriculture are frequently applied to various antibiotics during production processes (Done et al., 2015). Therefore, the demands of antibiotics constantly increase to meet the needs of the medical and production (Procópio et al., 2012). However, almost all antibiotic applications are beyond government monitoring, especially in developing countries (Aly and Albutti, 2014). Furthermore, antibiotics' limited biodegradability has posed major issues. Synthetic antibiotics, such as fluoroquinolones, are resistant to biological degradation (Sarangapani et al., 2019; Van Doorslaer et al., 2014). Therefore, the discharged wastewater from manufacturing processes for antibiotics production or hospital effluents contains high concentrations of antibiotics because biological wastewater treatment facilities are not principally able to achieve the sufficient removal of antibiotics (Booth et al., 2020, Kebede et al., 2019).

The spread of antibiotic-resistant microorganisms is well established to be the greatest hazard to human health (Amarasiri et al., 2020). In addition, antibiotics induce disturbances for the ecosystem. Specifically, many eukaryotes, such as phytoplankton and zooplankton in aquatic ecosystems, are directly and indirectly affected by antibiotics (Aderemi et al., 2018; Gorokhova et al., 2015; Lu et al., 2019; Motiei et al., 2020), as well as the direct effect of disturbance to the aquatically bacterial community (Eckert et al., 2019). Hence, antibiotics pollution is a currently big concern for human and environmental health (Polianciuc et al., 2020).

Specifically, synthesized antibiotics, fluoroquinolones such as ciprofloxacin (CFX) and ofloxacin (OFX), have been known for their very low biodegradability (Li and Zhang, 2010). Hence, they have a long life in aquatic environments (Janecko et al., 2016). CFX was detected in tremendous high concentrations (31,000 μg L^{-1}) in the effluent of the pharmaceutical wastewater treatment plant (WWTP) (Larsson et al., 2007). Besides, around 5,000 μg L^{-1} of CFX was also found in the municipal and pharmaceutical WWTP effluent and the surrounding river (Gothwal and Shashidhar, 2017; Hussain et al., 2016). On the other hand, a higher concentration than 3,000 μg L^{-1} of OFX was exposed in the effluent of the pharmaceutical WWTP (Hussain et al., 2016). Other survey results of CFX and OFX detected in various environmental matrices are shown in Table A-4 (Supplementary material). Furthermore, since CFX and OFX are reported to be high global consumptions (Bortone et al., 2021; Hsia et al., 2019) and are among the most used antibiotics in poultry and aquaculture industries (Kalunke et al., 2018; Xu et al., 2020), pollution of CFX and OFX is tremendous and continuous risks to water environments.

Ecotoxicologists who conducted a 21-day conventional toxicity test on sensitive freshwater creatures (e.g., *Daphnia magna*) recently expressed a strong interest in CFX (Dalla Bona et al., 2015; Dionísio et al., 2020; Martins et al., 2012; Motiei et al., 2020; Nunes et al., 2018). The inhibition of symbiotic microflora was identified to be one of the mechanisms impacting *D. magna* (Motiei et al., 2020) and damage to *D. magna* cells expressed by oxidative stress (Dionísio et al., 2020; Nunes et al., 2018)

and DNA lesions (Nunes et al., 2018) at environmentally relevant concentrations. On the other hand, to set environmental health standards, some studies have been performed to determine the median effective concentration (EC_{50}) of CFX for *D. magna* life-history traits, namely fertility (Dalla Bona et al., 2015; Martins et al., 2012). The obtained EC_{50}(s) of 12,800 and 24,000 μg L^{-1} of CFX were obviously higher than CFX residues detected in aquatic environments. In the above-mentioned studies, *D. magna* fertility was often quantified as summations of the entire test period (Dalla Bona et al., 2015; Martins et al., 2012; Nunes et al., 2018). Therefore, it is thought that fertility characteristics under antibiotic toxicities could be overlooked (Eltemsah and Bøhn, 2019). Besides, Motiei et al. (2020) expected the 21-day test could induce adverse effects of CFX on *D. magna* fertility; however, opposite results were obtained. In fact, the primary reproductive stage of *D. magna* is much longer than 21 days (Li et al., 2017). Therefore, the 21-day standard test perhaps generates an incomplete picture of the potential toxicities of CFX on *D. magna* fertility (Eltemsah and Bøhn, 2019; Nunes et al., 2018). Thus, to clarify characteristics of *D. magna* life-history attributes under the toxicities of CFX, a protracted exposure test is required.

On the other hand, considering the massive global usage and identified amounts in the environment, an ecotoxicological test for OFX is necessary. Despite chronic studies reported for OFX using several organisms, such as microalgae (Carbajo et al., 2015; Ferrari et al., 2004), diatom, blue-green algae, and crustacean (Ferrari et al., 2004), the chronic toxicity of OFX to *D. magna* remains unknown.

This research aims to elucidate the responses of the life history of *D. magna* at detected concentrations of CFX and OFX in environments during 42 days. In particular, the chronic toxicity of OFX to *D. magna* was initially described in comparison to CFX. In addition, we applied a generalized linear mixed-effects model (GLMM) as a more rigorous statistical analysis for ecotoxicological data to clarify the effect of CFX and OFX on *D. magna* life-history traits.

5.2. Material and methods

5.2.1. Reagents and stock preparation

Ciprofloxacin (CAS number: 85721-33-1) and ofloxacin (CAS number: 82419-36-1) powders with a purity of 98% or higher were procured from LKT Laboratories Co., Ltd. (USA) and Fujifilm Wako Pure Chemical Industries, Ltd. (Japan), respectively. Stock solutions of the antibiotics were prepared separately by dissolving them in pure water (Elga® PURELAB® Water Purification System, United Kingdom) at the final concentration of 1 g L^{-1}. Next, a few drops of 1 M HCl were added to the stock solution of 100 mL to completely dissolve the antibiotics powder under acidic conditions (pH 2–3), and then 1 M NaOH was added to neutralize the stock solution again. The stock solution was stored at 4 ºC in the dark during use.

5.2.2. Test organism and culture maintenance

The samples for the tests were collected from Japan's National Institute for Environmental Studies. *D. magna* populations from a clone were maintained in 1-liter beakers containing the culture medium of 1000 mL, which contained $CaCl_2{\cdot}2H_2O$ (294 mg L^{-1}), $MgSO_4{\cdot}7H_2O$ (123 mg L^{-1}), $NaHCO_3$ (64.8 mg L^{-1}), and KCl (5.75 mg L^{-1}) (ISO 6341, 2012). The following protocol, adapted from the USEPA

(2002), was used to make the mixed food for *D. magna*. Baker's yeast powder (Nissin, Japan) and fry's food powder (Sera Micron, Germany) was mixed with the gram ratio of 2 (food) :1 (yeast). From the manufactures data, the constituents of the mixed food in 1 gram were approximately calculated as follows: protein 0.41 g, carbohydrate 0.32 g, lipids 0.07 g, minerals 0.07 g. Then, the mixed food of 0.5 g was dissolved into pure water of 100 mL with ultrasonication (5 min). It was left to stand for 3 minutes to separate large particles, after which the supernatant suspension was collected. The mixed food stock contained 4.5–5.0 g total solid L^{-1} was stored at 4 ºC and the stock was used within 10 days.

D. magna was cultivated in a 1-liter beaker by adding the mixed food suspension of 1 mL once daily under the condition that 16 h light (700–800 lux) /8 h dark and the temperature of 20 ºC. The stock population of adolescent *D. magna* was isolated and cultivated until the *D. magna* became gravid females before the toxicity test. The offspring of those gravid females were traced down and used in the ecotoxicological experiment.

5.2.3. Ecotoxicological test protocol of CFX and OFX

The stock solutions of CFX and OFX were added separately into the culture medium to obtain the final concentrations of 50; 500 and 5000 µg L^{-1} for each antibiotic, where each culture medium was denoted as C50, C500, C5000 for CFX, and O50, O500, O5000 for OFX. Simultaneously with the antibiotic exposure test, a control (CT) with no antibiotics was done. The concentration range of 50–5000 µg L^{-1} for CFX and OFX exposures was determined based on concentrations of CFX and OFX detected in the environments (Table A-4). Ten neonates within 24 h old from brood 2 to brood 6 were randomly collected from the healthy females described in section 5.2.2 and were individually transferred to a 50 mL flask containing 30 mL of the culture medium. The mixed food suspension (see section 5.2.2) was fed to *D. magna* with the amount of 0.12 mg total solid per day for an individual (the equivalent concentration of 8 mg total solid L^{-1}) following the modified method of USEPA (2002) and Bahrndorff et al. (2016). The light intensity, the photoperiod, and the temperature were the same as the culture maintenance. Toxicity test was performed by maintaining the pH and dissolved oxygen of the medium at the range of 7.73–8.10 and 8.14–8.35 mg L^{-1}, respectively. Each culture medium containing CFX and OFX at each exposure concentration was replaced with the new medium every other day. Although CFX and OFX concentrations did not change after 2 days, considering the low biodegradability of CFX and OFX (Dalla Bona et al., 2015; Dang et al., 2021; Lu et al., 2019; Kümmerer et al., 2000), we confirmed this matter under the identical conditions of the exposure test in the preliminary experiment (Table A-5).

The exposure test lasted for 42 days. The assessment endpoints were survival, maturity, fertility, and offspring degradation. The day of the first oogenesis event observed in a brood chamber was used to determine maturity. The day of the first neonatal occurrence in a flask was then documented. Also, to evaluate fertility, the number of healthy neonates per capita for each flask was counted, with healthy surviving neonates defined as moving infants with no aberrant morphology. Neonates were then extracted from each flask. The total number of cumulative neonates of the ten flasks for each treatment expressed the overview of the reproductive characteristics of *D. magna* under each condition. The brood size per capita in each birth was evaluated. Finally, to assess the offspring

degradation, each flask was classified into the following four types: (1) dead eggs, (2) dead neonates, (3) dead eggs and dead neonates, and (4) malformed neonates. Moreover, the empty broods, which mean no living neonates in a flask, were recorded. The observation to assess the offspring degradation was performed daily using Nikon Eclipse 50i microscope (Japan) equipped with Canon EOS 60D camera (Japan).

5.2.4. Data analysis

Statistical analysis was performed in R software combining with RStudio (Lucent Technologies, Inc., USA). Symbols of lowercases: a' ($p < 0.1$), a ($p < 0.05$), b ($p < 0.01$), c ($p < 0.001$) were used to demonstrate the type I error levels between antibiotic exposures and the control, whereas asterisks: *'($p < 0.1$), * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) were used for indicating the type I error levels between CFX and OFX exposures with the same concentrations. In all statistical tests in this study, $p < 0.05$ was selected as a statistical significance.

In this study, we performed four types of statistical analysis as described later. As post hoc analysis, it needed to achieve multiple comparisons for the following nine pairs in each analysis: CT–C50, CT–C500, CT–C5000, CT–O50, CT–O500, CT–O5000, C50–O50, C500–O500, and C5000–O5000 (Treatment = CT: control, C50: 50 µg CFX L^{-1}, C500: 500 µg CFX L^{-1}, C5000: 5000 µg CFX L^{-1}, O50: 50 µg OFX L^{-1}, O500: 500 µg OFX L^{-1}, O5000: 5000 µg OFX L^{-1}). Each raw *p* value between two comparative groups was generated in each analysis. Then, adjusted *p* values were calculated for raw *p* values using the Benjamini & Hochberg method (Waite and Campbell, 2006).

The four types of statistical analysis used in this study are as follows.

1) To analyze the day of the first oogenesis or neonates, we elected a nonparametric statistical model for hypothesis testing due to the lack of underlying probability distribution for the random variable (day) (Schober and Vetter, 2020). First, the Kruskal-Wallis H test was applied, followed by Dunn's test ("dunn.test" package).

2) This study, used a Poisson-based generalized linear mixed-effects model (GLMM) ("lme4" package) to assess brood size (Bolker et al., 2009), using the number of neonates as the objective variable and the following explanatory variables as fixed effects: Birth sequence of broods (Birth = birth 1, birth 2,..., birth 10) as an ordinal variable, treatment with the seven exposure conditions indicated above as a categorical variable, and exposure time (Time = days after the start) as a continuous variable. The random effect (1|replica) corresponds to the differences among replicates and thus it deals with the problem of overdispersion. The global model was demonstrated by the following equation (1).

$$\text{Brood size} \sim \text{Treatment} + \text{Birth} + \text{Time} + [\text{Interactions}] + (1|\text{replica}) \quad (1)$$

[Interactions] are the interaction terms for all combinations of Treatment, Birth, and Time variables (see Table S2).

Conditional Akaike Information Criteria (cAIC) were used to compare the GLMMs with various combinations of fixed effect variables, including interaction terms (Table S2). A GLMM model with a lower cAIC means a better model (Vaida and Blanchard, 2005). In this study, to evaluate the correlation between a continuous variable (Time) and an ordinal variable (Birth), the

polyserial correlation was calculated based on the maximum likelihood estimates of correlation ("polycor" package).

3) GLMM was again applied to analyze the brood size among exposures in each birth (birth 1, birth 2,…, birth 10) using the following model as shown in equation (2), and we also evaluated the cAIC in each model.

$$\text{Brood size} \sim \text{Treatment} + \text{Time} + \text{Treatment} \times \text{Time} + (1|\text{replica}) \quad (2)$$

Where Treatment × Time indicates an interaction term.

To analyze progeny degradation, each count data for classified four types (see section 2.3) and empty broods were hypothetically examined by Fisher's exact test (Stoner et al., 2003).

5.3. Results

5.3.1. The effects of CFX and OFX on survival and maturity

Each individual remained alive during the exposure test. For each exposure condition, the first observed eggs in the *D. magna* brood chamber were used to determine maturity (the day of the first oogenesis). Then, neonates were first observed in the flask (the day of the first neonates). As shown in Fig. 5-1, statistical significance was only found for the day of the first oogenesis of C500 exposure earlier than the CT ($p = 0.019$) and the O500 exposure ($p < 0.001$). In contrast, the first oogenesis of C5000 exposure was nearly statistically significant earlier than the CT ($p = 0.079$). All conditions of OFX exposure did not show statistical significance compared to the CT, though the first oogenesis of O5000 exposure was nearly statistically significant earlier than the CT ($p = 0.063$). Both CFX and OFX exposures had no meaningful effect on first newborns at all doses (Kruskal-Wallis H, $\chi^2_{[6]} = 11.29$, $p = 0.08$).

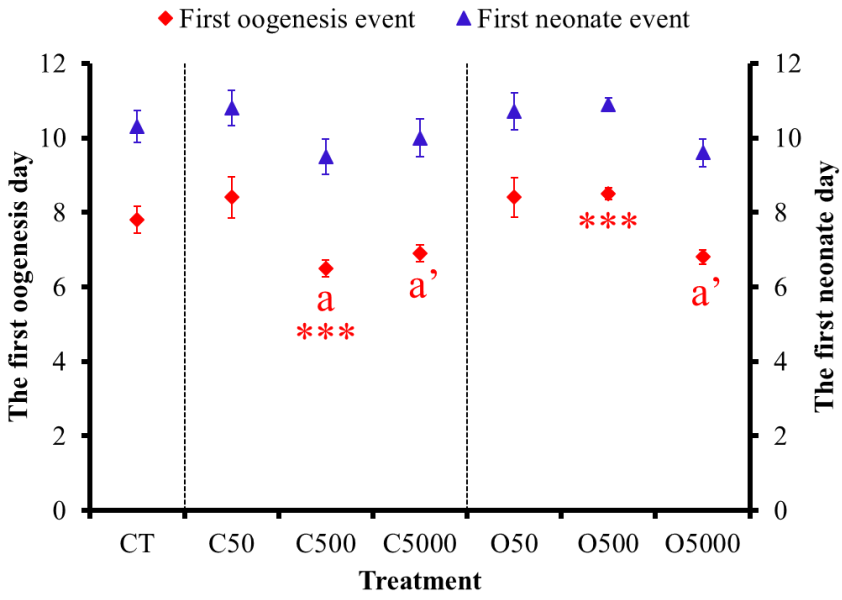

Fig. 5-1. First oogenesis and neonate days for the ciprofloxacin (CFX) and ofloxacin (OFX) exposures of various concentrations and the control (CT). C50, C500, C5000, O50, O500, O5000 denote CFX at 50, 500 and 5000 μg L^{-1}, and OFX at 50, 500 and 5000 μg L^{-1}, respectively. Each data is expressed as mean ± standard error.

5.3.2. The effects of CFX and OFX on fertility

Overview of the effect of exposure conditions, exposure time, and birth sequence

Fig. 5-2A showed temporal changes in the average number of neonates per capita for each exposure condition. This line graph fluctuated wildly, but it peaked around 2 weeks and 1 month after the start of exposure, and it seemed that the produced neonates tended to decrease according to the exposure time. Specifically, *D. magna* exposed to C500 had far more neonates than the control *D. magna* at about days 10–16, and C5000 and O5000 exposures had fewer neonates than the CT at about days 14–30. Smoother curves are sometimes seen in the cumulative curve of neonates by exposure time to suggest changes between antibiotic exposures and the CT (Fig. A-3). However, it is challenging to depict the effect of the exposure duration.

On the other hand, the two peaks are more precise for the plot of each brood size by the birth sequence (births 1–10), as shown in Fig. 5-2B. This is because the birth of *D. magna* was not perfectly synchronized; thus, the exact birth does not mean the same exposure time. However, each line of the brood size in Fig. 5-2B clearly shows the difference between antibiotic exposure conditions and the CT than in Fig. 5-2A. Besides, the CT has two peaks at birth 3 and birth 7, and each antibiotic exposure has two peaks at birth 2 or 3 and birth 7 or 8, respectively. The brood sizes of C50 and C500 were more extensive than the CT at birth 2, and the brood sizes of O50 and C50 were more prominent than the CT at birth 7. Then the brood sizes of C5000 and O5000 were consistently lower than the CT from births 3–9.

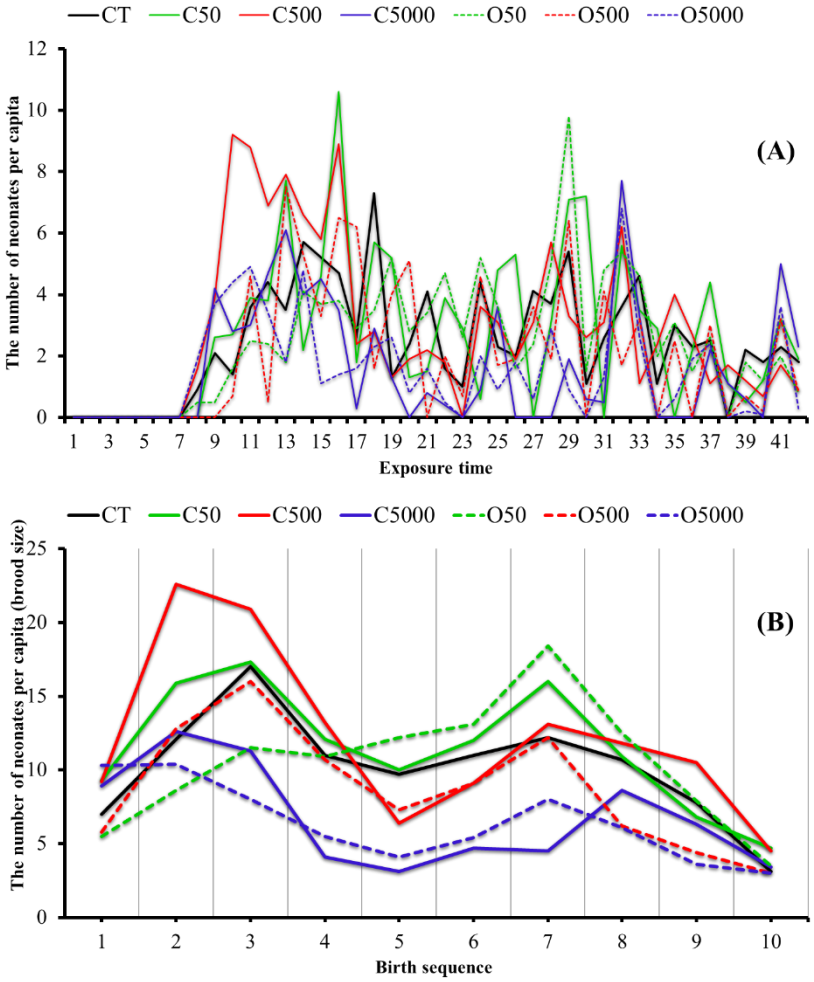

Fig. 5-2. Changes of the average number of neonates per capita in each exposure (C50–5000, O50–5000) and the control (CT) for the exposure time within 42 days (A) and the birth sequence till ten births (B).

Table 5-1. The summary table of GLMMs 12 and 9 presents the effects of explanatory variables (Treatment, Birth, and Time).

	Model 12: Brood size ~ Treatment + Birth + (1|replica)				**Model 9:** Brood size ~ Treatment + Birth + Time + (1|replica)			
	Estimate	Std. Error	z value	*p*(>|z|)	Estimate	Std. Error	z value	*p*(>|z|)
Treatment								
Intercept	2.13259	0.08883	24.009	< 0.001	2.985	0.1419	21.034	< 0.001
C50	0.12852	0.11124	1.155	0.248	0.1276	0.1008	1.267	0.205
C500	0.16959	0.11105	1.527	0.127	7.011×10^{-05}	0.1031	0.001	0.999
C5000	−0.43225	0.11431	−3.781	< 0.001	−0.4699	0.1043	−4.504	< 0.001
O50	0.02562	0.11167	0.229	0.819	−0.01909	0.1014	−0.188	0.851
O500	−0.12876	0.11248	−1.145	0.252	−0.1252	0.1021	−1.225	0.220
O5000	−0.47418	0.11605	−4.086	< 0.001	−0.4511	0.1060	−4.256	< 0.001
Birth								
2	0.52853	0.05318	9.938	< 0.001	0.7439	0.06084	12.227	< 0.001

3	0.59962	0.05250	11.421	< 0.001	1.061	0.08212	12.918	< 0.001
4	0.18678	0.05706	3.273	0.001	0.9138	0.1145	7.983	< 0.001
5	−0.05192	0.06056	−0.857	0.391	0.9491	0.1495	6.349	< 0.001
6	0.14668	0.05769	2.543	0.011	1.434	0.1849	7.757	< 0.001
7	0.41714	0.05442	7.665	< 0.001	2.005	0.2234	8.975	< 0.001
8	0.18327	0.05720	3.204	0.001	2.079	0.2647	7.853	< 0.001
9	−0.13615	0.06238	−2.183	0.029	2.077	0.3081	6.743	< 0.001
10	−0.36198	0.07622	−4.749	< 0.001	2.133	0.3489	6.114	< 0.001
Time					−0.08025	0.01096	−7.325	< 0.001

GLMM analysis for brood size in the whole period

For the brood size, GLMM by equation (1) was applied to illuminate the influence of exposure conditions, birth sequence, and exposure time. Table A-6 showed the comparative results of several GLMMs (model 1–14) using cAIC, deviance, and log-likelihood (logLik) to select a better model. All model has to contain Treatment variable and the random effect (1|replica). Model 1 showed the lowest cAIC, deviance, and logLik; therefore, it was the best model to explain or predict the brood size (objective variable). Model 2 was second best for lower deviance and logLik. However, since model 2 had a slightly higher cAIC than model 4, model 4 was the second-best model in cAIC.

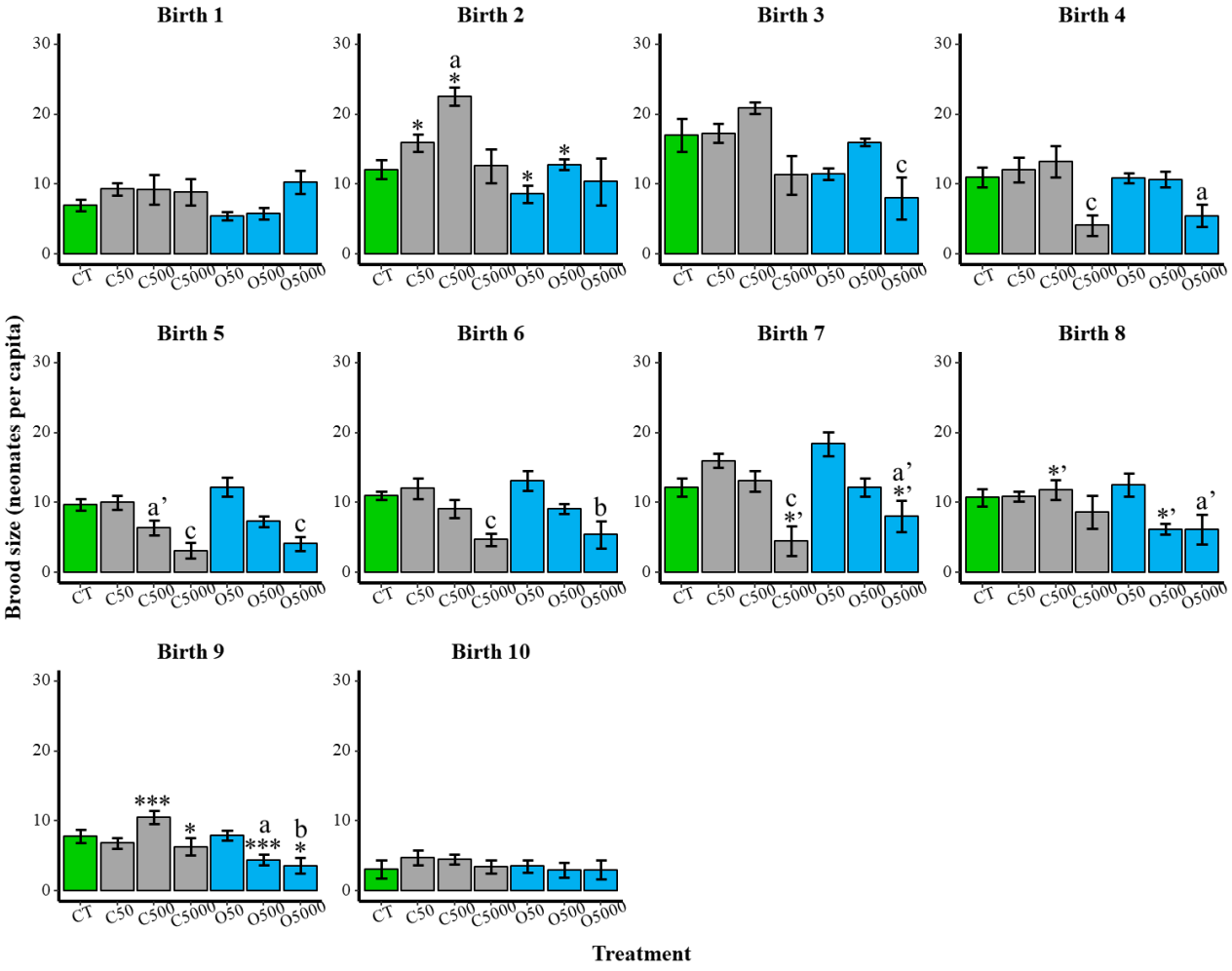

Fig. 5-3. The effects of exposure conditions (CT, C50–5000, O50–5000) at each birth in the birth sequence on the *D. magna* brood size. The bar graphs are expressed as mean ± standard error.

According to the observation of changes in cAIC in Table A-6, exposure time had a lower significant effect on the brood size compared to the birth sequence. The cAIC of model 10 indicated this because the cAIC of model 10 was next smaller than model 4, and the cAIC of model 11 was much larger than model 10.

Table 5-1 showed the results of model 12 and model 9, which did not include the interaction term. Before referring to Table 1, we suggest the lower cAIC of model 12 compared to model 9 even though

collinearity between Birth and Time variables is highly potential. This matter will be clarified in the discussion part (see section 4.6). Estimates in Table 1 correlated to logarithmically transformed brood size (log-brood size). In model 12, the intercept estimate corresponded to the CT's log-brood size at birth 1. Each estimate of C50–O5000 was the relative value from CT. Thus, only C5000 and O5000 significantly reduced the fertility of *D. magna* ($p < 0.001$).

Besides, each estimate of births in model 12 (2–10) was also relative to the first birth and was statistically significant ($p < 0.05$) in all cases except birth 5 in model 12. Thus, model 12 in Table 1 clearly showed the peak of brood size at births 2 and 3 and the peak at birth 7, as shown in Fig. 2B. Nevertheless, estimates of model 9 had to be always subtracted by the effect of the Time variable, i.e., −0.08025 × (average exposure time of each birth), to compare the estimates of model 12.

GLMM analysis on brood size in each birth

From Table A-6, we judge that birth sequence significantly interacts with exposure conditions (model 10). However, we do not discover directly the effects of exposure conditions on the brood size at each birth using the interaction term of "Treatment × Birth", which can be supplied completely in GLMM outputs. In detail, the argument of this issue was provided in the discussion part (see section 5.4.6). Therefore, GLMM analysis by equation (2) was independently applied to brood size in each birth. First of all, the model selection using cAIC has applied the model for each birth. The results showed the lowest cAIC for the simplest model that "Brood size ~ Treatment + (1|replica)" except for the birth 9 (Table A-7). Besides, there was no effect of exposure conditions on exposure time at each birth (Fig. A-4). Therefore, the simplest GLMM was adopted. In birth 1, there was no effect from exposure conditions on the brood size (GLMM, $\chi^2_{[6]} = 12.46$, $p = 0.052$) (see Fig. 5-3). In birth 2, the brood size of C500 exposure showed a significantly larger than those of CT and O500 exposure. Besides, the brood size of O50 exposure was significantly smaller than that of C50 exposure. From births 3 to 6, *D. magna* exposed to O5000 significantly reduced brood size than the control *D. magna*. On the other hand, the brood size of C5000 exposure was significantly lower than CT from birth 4 to 7. In birth 5, the smaller brood size for C500 exposure compared to CT showed a $p = 0.08$. In birth 7, the brood size of O5000 exposure was smaller than that of CT and was larger than that of C5000, but not significant ($p = 0.093$ and $p = 0.094$, respectively). In birth 8, the brood size of O5000 exposure was smaller insignificantly than that of CT with a $p = 0.076$. Furthermore, the brood size of O500 exposure was smaller than C500 with a $p = 0.064$. In birth 9, the brood sizes of O500 and O5000 exposures were significantly smaller than CT. Besides, each brood size of C500 and C5000 exposures was significantly larger than those of O500 and O5000 exposures, respectively. Finally, in birth 10, there was no significant effect from the exposure conditions on the brood size (GLMM, $\chi^2_{[6]} = 4.4643$, $p = 0.614$).

5.3.3. The effects of CFX and OFX on offspring degradation

Each type of offspring degradations

Images A–E in Fig. A-5 presented 4 types of offspring degradation, and image F shows a typical healthy living neonate of the CT. The round shape was a type of dead eggs as shown in Fig. A-5A,

and then the bumpy shape was a type of a dead *D. magna* neonate as shown in Fig. A-5B. Fig. A-5C showed both in a flask. Malformed *D. magna* individuals such as Fig. A-5D and A-5E could be easily identified by anomalous morphology from the healthy *D. magna* of Fig. A-5F.

Fig. 5-4A and 5-4B showed the proportion of flasks with degraded offspring in each birth and exposure condition. It was exhibited that CT resulted in no degraded offspring in all births, but the percentages of flasks with degraded offspring were 10% of C50, O50, C500, and O500 exposure at several births. C5000 and O5000 exposure resulted in a high likelihood of producing degraded offspring, up to 90% for CPX and 70% for OFX, especially at births 6 and 7.

Fig. 5-5A and 5-5B showed the proportion of flask containing each type of offspring degradation in each birth under C5000 and O5000 exposures. The proportion of flasks with dead eggs of C5000 exposure was very high throughout most of the births, whilst a flask with dead neonates was found at births 3 and 7, and a flask with both dead eggs and neonates was found at birth 7. On the other hand, the proportion of flasks with dead eggs of O5000 exposure was also high throughout several births. Besides, a flask and 2 flasks with dead neonates and malformed neonates, respectively, were found birth 3, followed by flasks with dead eggs and neonates at births 4, 5, and 7–9. Furthermore, in Fig. 5-5A and 5-5B, the proportions of C5000 flasks with empty broods were higher than O5000 exposure at births 5 and 7, but slightly lower than O5000 exposure at birth 6.

The scatter plot (Fig. A6) presented the relationship between the relative total healthy neonates of *D. magna* nourished in C5000 and O5000 exposures and the proportion of maternal *D. magna* (flasks) producing offspring degradation. We expected that Fig. A6 showed a negative correlation that the relative healthy neonates declined until the proportion of *D. magna* producing the offspring degradation was about 30%. However, as over 50%, no apparent change in that observation was found.

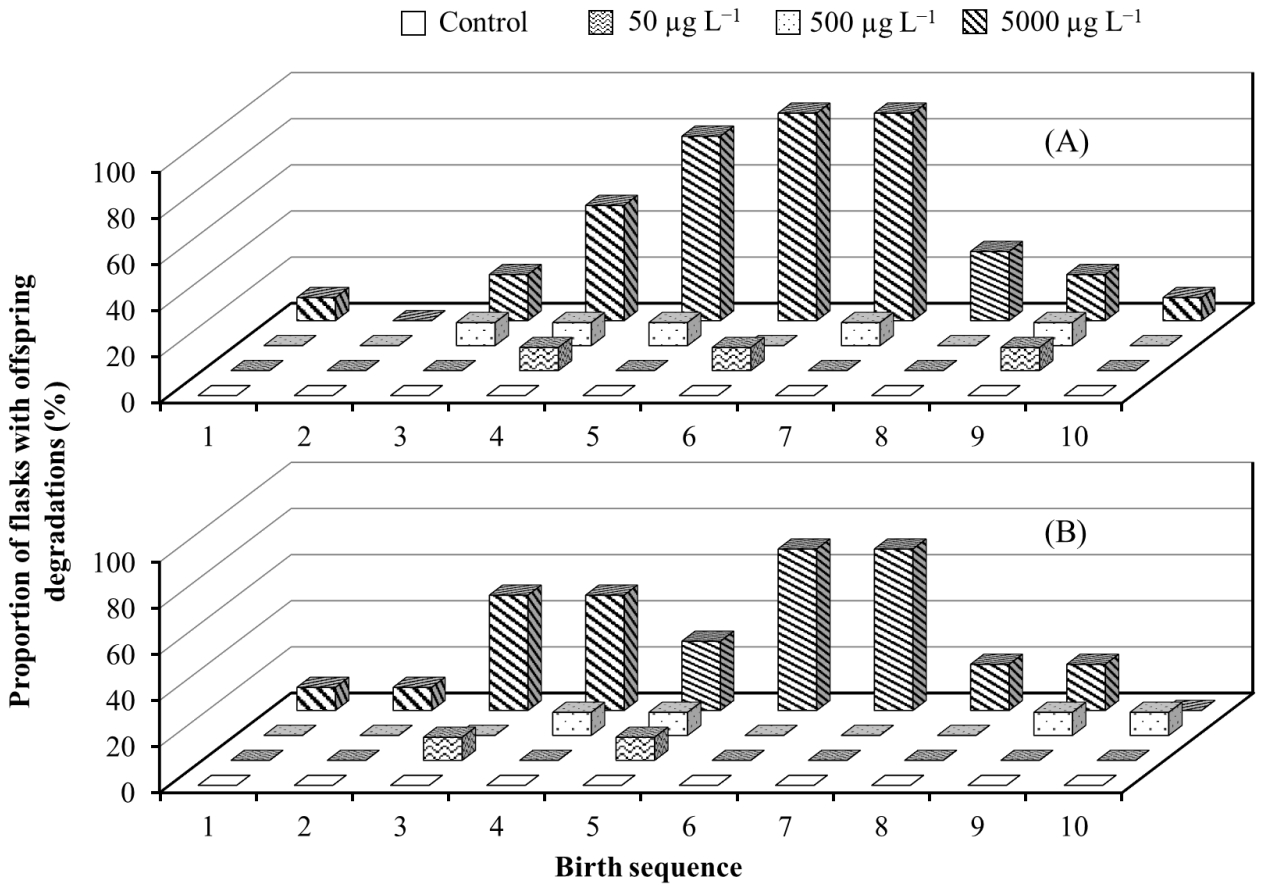

Fig. 5-4. The proportion of flasks with offspring degradations (ten flasks considered as 100%) under the effects of exposure conditions CFX (A) and OFX (B), and birth sequence.

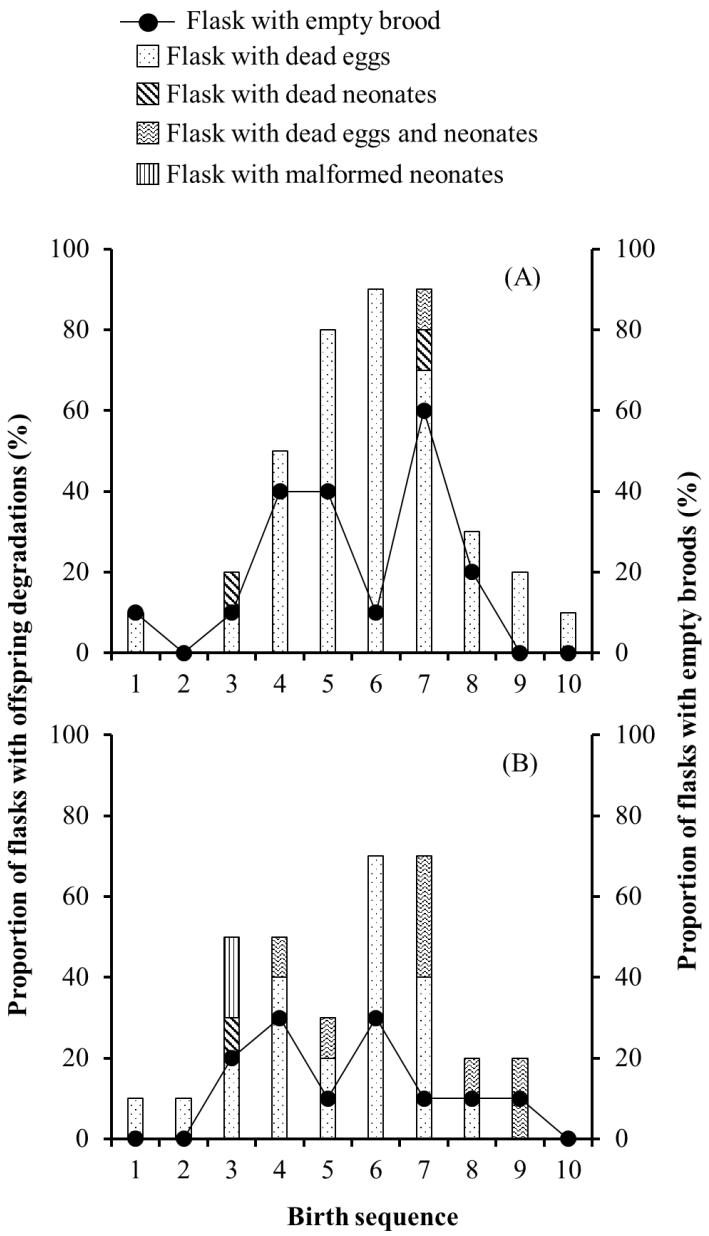

Fig. 5-5. The proportion of flasks with offspring degradations in each type and the proportion of flasks with empty broods were analyzed for *D. magna* nourished in the antibiotic exposure at 5000 μg L^{-1}. Note that the malformed neonates are always associated with dead eggs and neonates, and empty broods are always associated with offspring degradations.

Statistical test of offspring degradation by the exposure conditions

Table 5-2 presented the enumeration of the flasks with each type of offspring degradation and empty broods from births 1–10. The C50, C500, O50, and O500 exposures showed no empty brood and a couple of the flasks with offspring degradation, which were not significantly different in all pairwise comparisons. C5000 and O5000 exposures showed significant differences in the number of the flasks with dead eggs from CT and were more in C5000 exposure than that of O5000 exposure, but not statistically significant ($p = 0.088$). We did not find statistical significance in all pairwise comparisons for dead neonates, dead eggs and neonates, and malformed neonates. Although O5000 exposure showed 8 flasks totally but was insignificantly different from CT ($p = 0.061$) in the case of offspring degradation by dead eggs and neonates. Besides, only C5000 and O5000 exposures showed a significant difference from CT for the flasks with empty broods.

Table 5-2. The enumeration of the flasks with each type of offspring degradations and the flasks with empty broods from births 1–10.

	Flask with dead eggs	**Flask with dead neonates**	**Flask with dead eggs and neonates**	**Flask with malformed neonates**	**Flask with empty brood**
CT	0	0	0	0	0
C50	3	0	0	0	0
C500	3	2	0	0	0
C5000	$37^{c,*'}$	2	1	0	19^{c}
O50	1	1	0	0	0
O500	2	2	0	0	0
O5000	$22^{c,*'}$	1	$8^{a'}$	2	12^{b}

Note that:

The malformed neonates are always in association with dead eggs and neonates

Empty broods are always in association with offspring degradations

5.4. Discussion

5.4.1. Effects of CFX and OFX on the mortality of *D. magna*

Concentrations of CFX and OFX examined in this study did not cause mortality for *D. magna*. The expected result for CFX exposures is because Dalla Bona et al. (2015) reported that a CFX concentration of 15 mg L^{-1} was considered not to reduce *D. magna* survival in the 21-day chronic test. On the other hand, exposure to OFX did not cause mortality for water flea *Ceriodaphnia dubia* at 10 mg L^{-1} after the 7-day chronic test (Ferrari et al., 2004). Therefore, 100% alive *D. magna* in our study until the end is quite understandable and this result is also the first report for *D. magna* survival under prolonged exposure to OFX. As a result, we used reproduction-related endpoints to elucidate antibiotic effects in our investigation. Reproduction is an important trait of herbivorous zooplankton linked to population dynamics and possibly impacts community organization (Adamczuk et al., 2020; Eltemsah and Bøhn, 2019).

5.4.2. The endogenous character of the *D. magna* fertility

Before the discussion of the toxicity effects of CFX and OFX, it should be noted that the brood size (fertility) changes with two peaks, as shown in Fig. 5-2, concerning the birth sequence (also exposure time), even in the control system. Thus, these two peaks are independent of the effects of antibiotic exposures. Furthermore, this intrinsic property of *D. magna* fertility has already been demonstrated in studies on its lifespan (Anderson and Jenkins, 1942; Enserink et al., 1990; Gustafsson et al., 2005). Thus, it is judged that the CFX and OFX exposures modified the inherently endogenous properties of *D. magna*.

5.4.3. The positive effect of CFX (C500) on the first oogenesis and the brood size

One of the most interesting results is that a shortening of the first oogenesis date was caused by C500 exposure, though it was not affected by C50 and C5000 exposures and all OFX exposures, as shown in Fig. 5-1. On the other hand, the date of the first neonates was not statistically different among exposure conditions. For example, Martins et al. (2012) reported the significantly longer date of the first neonate under extremely high CFX exposure at 25.5 mg L^{-1} and no change in that endpoint until 1.79 mg L^{-1}. Meanwhile, Motiei et al. (2020) and Nunes et al. (2018) did not detect any change in the first neonate date among CFX exposures at 5–1,000 μg L^{-1}. However, the previous studies on the chronic toxicity of CFX did not report the changes in the first oogenesis date for *D. magna*. Therefore, our study is the first report for such an endpoint. In addition, it is fascinating that the C500 exposure involved the statistically significant increment ($p < 0.05$) of the brood size in the second birth. Motiei et al. (2020) also reported that the *D. magna* brood sizes were increased considerably within 21 days when exposing *D. magna* to CFX concentrations of 10; 100; and 1,000 μg L^{-1}.

The shortening of the first oogenesis date and the increment of brood size can suggest the phenomenon known as hormesis (hormetic effects), by which toxins or stressors give positive or favorable effects to organisms compared to a normal state of the organism (Calabrese, 2008; Calabrese and Baldwin, 2003; Calabrese and Mattson, 2017). The possible mechanism involved in an increment of energy consumption to protect cells against oxidative stress at low CFX

concentrations of 0.5–1,000 μg L^{-1} could cause overcompensation (Aderemi et al., 2018; Dionísio et al., 2020; Motiei et al., 2020; Nunes et al., 2018). The overcompensation for stress is considered an important mechanism of hormetic effects (Calabrese, 2001). In another scenario, although study results of Motiei et al. (2020) suggested that microbiome-mediated effects are less important than the direct effects to cause hormesis, their results also revealed that the abundances of *Bosea* and *Galbitalea* genus in the gut of *D. magna* responsible for filtered food degradation, increased by CFX exposures. Thus, microbiome-mediated effects should not be excluded as the root of hormesis.

On the other hand, unlike the CFX, the O500 exposure did not significantly affect maturity and brood size at the second birth. Since a lack of chronic tests for OFX using *D. magna* in literature, our result is the first report for OFX toxicities with *D. magna*. Dalla Bona et al. (2015) mentioned the positive relation between hydrophobicity and toxicity. Accordingly, since the hydrophobicity of OFX (log $K_{ow} = -0.39$) is lower than CFX (log $K_{ow} = 0.28$), OFX is harder to cross the cell membrane than CFX (Turek et al., 2020). In addition, taking into account molecular structure, it is also suggested that the biochemical effects of OFX on a cell are weaker than those of CFX (Peterson, 2001).

5.4.4. Reduction of the brood size and offspring degradation with the CFX and OFX exposures

Fluoroquinolones interact with topoisomerase II and DNA to form the ternary complex, inducing the replication process's blockage in eukaryotic cells (Fief et al., 2019). Recent ecotoxicological research by Lu et al. (2019) and Nunes et al. (2018) indicated that the CFX suppresses gene functions such as nucleoids, transcription, and translation in eukaryotic organisms' cells, including *D. magna* at 7–195 μg L^{-1}. However, our results and other ecotoxicological studies (Dionísio et al., 2020; Motiei et al., 2020; Nunes et al., 2018) did not show the negative effects of CFX on *Daphnia* life-history traits and behaviors at their concentrations, even up to 1,000 μg L^{-1}. This is a gap between individual- and molecular-level phenomena, but no researcher explained the reason for the gap plausibly. On the other hand, as already discussed, fluoroquinolones are also the pro-oxidant and the known subsequent outcome of oxidative stress is genotoxicity (Nunes et al., 2018). Thus, C5000 and O5000 exposures are not excluded from causing high pressures for unrepaired DNA lesions because high levels of oxidative and replicative stresses have a synergistic interaction to increase the probability of DNA lesions (Mouw et al., 2017). Accordingly, maternal DNA lesions cause to reduce the offspring viability of the eukaryote in the toxicity test (Nava-Rivera et al., 2021). Thus, we considered that offspring degradation and the decreases of brood sizes at C5000 and O5000 exposures were possibly associated with the DNA lesions of *D. magna.* In our results, Fig. 5-4 (also Fig. 5-5) and Table 5-2 showed the offspring degradation by C5000 and O5000 exposures to cause the brood size reduction (Fig. 5-3). As a proof, Mu and LeBlanc (2002) reported that the decrease of offspring viability highly correlated to the *D. magna* fertility when exposing *D. magna* to fungicide fenarimol. Our study is the first report on the offspring degradation under fluoroquinolone effects.

Many researchers in ecology and ecotoxicology have currently agreed on the crucial roles of the gut microbiome for the impacts on host fitness (Akbar et al., 2020; Cooper et al., 2021; Gorokhova et al., 2015). In Cooper and Cressler (2020) discussion, we found that essential amino acids for *D. magna* developments and fertility are derived from several bacterial taxa. Besides, based on antimicrobial susceptibility testing of CFX and OFX (Hoogkamp-Korstanje, 1997; Sueke et al., 2010), it was

considered that the high concentrations of CFX and OFX in this study (i.e., 5,000 μg L^{-1}) resulted in the non-selective suppression of gut bacterial communities in *D. magna*. Consequently, undernourishment of maternal *D. magna* resulted in offspring degradations and brood size reduction (Bednarska and Slusarczyk, 2013; Eltemsah and Bøhn, 2019). Thus, we hypothesized that the suppression of gut bacterial populations could account for offspring deterioration and fertility loss in our work.

5.4.5. Adaptation of *D. magna* exposed to CFX and OFX

The proportions of offspring degradation were sharply decreased at birth 8–10 of C5000 and O5000 exposures as shown in Fig. 5-4 and Fig. 5-5. In addition, Fig. 5-3 revealed that there was no significant difference in the brood size between CT and C5000 exposure at births 8–10. These findings were attributed to *D. magna*'s adaptation throughout the protracted exposure test. Such modifications were not visible in 21-day experiments, highlighting the necessity of our longer investigation in determining the CFX toxicity. However, the adaptation to OFX expressed by the brood size was not obtained.

Given that it takes about a month to adapt, the mechanism of adaptation may be related to bacterial resistance developed in the gut microbial community to fluoroquinolones (Redgrave et al., 2014). On the other hand, Motiei et al. (2020) supposed that the adaptation of the host is like to reflect the recovery of the gut microbiome in antibiotic exposure tests. Therefore, we need a further study on the prolonged effects of CFX and OFX on the gut microbial community to clarify this relationship.

5.4.6. Importance of statistical method in this study

We would like to have a supplementary discussion on GLMM. First, we need to mention collinearity between birth sequence (Birth: ordinal categorical variables) and exposure time (Time: continuous variables). Typically, to evaluate the collinearity problem, VIF (variance inflation factors) is used with the definition as follows: $VIF = 1/(1 - r^2)$, where r means polyserial correlation (O'brien, 2007). Each polyserial correlation between birth sequence and exposure time of each exposure condition is about 0.953–0.999 resulting in the VIF is about 21–1000. If VIF > 10, that collinearity needs to be considered (Mouillot et al., 2011). When both variables with large VIF participated in a regression model, cAIC can generally increase due to instability in parameter estimation (Heino et al., 2017). However, in our case, when the exposure time is removed from model 9, cAIC of model 12 increases. Therefore, in this analysis, the issue of collinearity can be ignored. VIF is not adequate as the indicator of collinearity issue in the correlation between an ordinal categorical variable and a continuous variable. Future studies need to be implemented to clarify this statistical problem. Second, model 10, which stresses the interaction of Treatment and Birth variables, should be utilized to elucidate the effect of exposure circumstances on brood size at each birth. Unfortunately, the statistical inference of GLMM outputs for model 10, which includes categorical variable interaction, must have a large number of coefficients, which is a practical challenge. Specifically, the interaction term "Treatment (7 categories) × Birth (10 categories)" produces 70 of compared pairs, which makes it very difficult to discuss and interpret the results in this paper. Moreover, it is infrequent to be statistically significant in those pairwise contrasts because there are too many simultaneous comparisons (Albers, 2019).

Therefore, this study focused on the multiple comparisons within each birth as described in section 5.2.4 (equation 2) (Imhof et al., 2017). Despite these disadvantages of unsolved problems present in our case, GLMM effectively demonstrates the importance of each factor and interaction in this study. We highly recommend GLMM to be an essential candidate for longitudinal data analysis, which should be applied for long-term, transgenerational, and multigenerational experiments. Therefore, future research needs to develop ideas for deriving complete information on the linear model that contains categorical variables and their interactions. As a priority, GLMM analysis in the Bayesian framework may study to compromise the complicating problems recognized in our analysis (Fong et al., 2010).

5.5. Conclusion

This study conducted a 42-day ecotoxicological test of CFX and OFX to *D. magna*. No mortality for *D. magna* was observed in the control and all antibiotic exposure conditions until the end of the exposure test. The earlier maturation and the increment of brood size were expressed in the C500 exposure. We considered that these phenomena linked to hormetic effects. Statistical analysis based on GLMMs revealed significant reductions of brood size in C5000 and O5000 exposures. At this concentration of CFX and OFX, offspring degradation such as dead eggs was also found. In addition, we found that the CFX and OFX exposures showed the adaptations in offspring degradation and the brood size at births 8–10, but the OFX did not show the explicit adaptation for brood size. More research is needed to better understand the mechanisms of CFX and OFX adaptative responses, particularly from the perspective of the long-term effects of CFX and OFX on the gut microbiota of *D. magna*. This study results demonstrate the effectiveness of GLMMs as a powerful and rigorous statistical analysis tool and the need for new statistical methods.

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6. SUMMARIES AND FURTHER WORKS

6.1. Summaries

Chapter 3: In summary, *D. magna* survival rate was insignificantly reduced by both CCEs. Besides, the fertility of *D. magna* exposed to both CCEs was drastically improved, even at the lowest concentration. Although the reproduction-stimulated effects on *D. magna* were expressed obviously at evaluated endpoints, the offspring-toxic effects were also recorded in all MCCE and NCCE exposures. Besides, *D. magna* somatic growth was also increased under exposures to MCCE and NCCE at all the tested concentrations. Interestingly, the feeding rate was only enhanced by MCCE exposures, which indicated that some dissimilar properties between CCEs remained unknown. Here, our results were new and interesting that ecotoxicologically indicate the feeding rate as well as life-history trait responses of *D. magna* to MCCE and NCCE at its early stage of development. The toxicities of *Pseudanabaena* sp. extract on *D. magna* obtained in our study were considered as the first report for further investigations. Ecotoxicological tests with reservoir water collected directly from where cyanobacterial blooms occurred by using *D. magna* and/or the variety of species will be investigated in the future.

Chapter 4: No mortality of the tested organism was observed in the tests of CFX, OFX, DFX, TET, and MIX, whereas only 10 and 0% of the total number of tested organisms were alive in GFX exposures of 20 and 40 mg L^{-1}, respectively. In the GFX test, except for the exposures of 20 and 40 mg L^{-1} (due to the lethal effect), significant reductions of *S. vetulus* TLR were observed in the exposures of 2.5–12 mg L^{-1}. On the other hand, we found the hormesis effects on *S. vetulus* TLR when exposing the organisms to 10–40 mg L^{-1} for CFX, and to 1.25–40 mg L^{-1} for OFX. In DFX and TET tests, we did not detect any significant effect of antibiotic exposure conditions on *S. vetulus* TLR. In the test of the antibiotic mixture, the highest concentration of pooled five antibiotics (total antibiotic concentration of 40 mg L^{-1}) decreased significantly TLR of exposed organisms. Our obtained results are considered as the first report for the acute effects of single antibiotics and antibiotics in the mixture on *S. vetulus* physiological parameter. Besides, our results show unpredictable toxicities of fluoroquinolone antibiotics and antibiotic mixture. Thus, our study supported the preliminary results for further works which are highly recommended to clarify the different mechanisms of action between fluoroquinolone antibiotics as well as antibiotic–antibiotic interactions in the mixture. We believe that TLR endpoint is a useful tool for screening investigations of toxic levels of interested toxicants, especially antibiotics or pharmaceuticals which often possess low intrinsic toxicities to non-target organisms. Therefore, researchers can save materials used for the study. Besides, we also suggest that *S. vetulus* is highly compatible with the evaluation of TLR endpoint because of its natural character of immobility and the absence of the jumping-like movement (in *D. magna*). Therefore, we do not need to use additional chemicals (e.g. glue) which require additional expense and potentially generate questionable results. However, to avoid confounding effects on experimental results when using *S. vetulus* as model organisms in ecotoxicological tests, the selection of neonates based on maternal age (birth or time) for the test needs to be carefully considered, and therefore the request for a standard guideline is necessary.

Chapter 5: No mortality for *D. magna* was observed in all exposure conditions of CFX and OFX. The statistical analysis results for the endpoints did not present significant effects of the exposure of

CFX and OFX at 50 µg L^{-1} on *D. magna* maturity and fertility. Therefore, this study recommends that life-cycle tests be performed to achieve significant results at a low concentration of 50 µg L^{-1} under the diagnoses of *D. magna* life-history traits. On the other hand, although the strong hormesis effects on maturity and fertility were expressed in the CFX exposure at 500 µg L^{-1}, the OFX exposure at 500 µg L^{-1} did not demonstrate the adverse effects on *D. magna* fertility. GLMMs based on Poisson distribution considering overdispersion of brood size revealed that the reduction of brood size was significant for the exposure of CFX and OFX at 5000 µg L^{-1}. The statistically significant offspring degradations were seen as maternal *D. magna* exposed to CFX and OFX of 5000 µg L^{-1}. In addition, both exposures showed the recovery or the adaptation in the fertility of *D. magna* during the long exposure period. The production of dead eggs as offspring degradation in the CFX exposure was greater than in the OFX exposure, and it implied the higher toxicity of CFX than OFX which can be explained with fluoroquinolone toxicity mechanism based on the molecular cell biology as direct effect and the symbiotic microbiome in the gut of *D. magna* as an indirect effect. However, currently known mechanisms alone have not provided sufficient explanation for the results that OFX exposure at 5000 µg L^{-1} showed a long-lasting reduction effect compared to CFX exposure. This study is the first to show the results of an OFX exposure test compared to CFX exposure. Therefore, our research provides new knowledge and new guidelines in future studies for assessing these antibiotic contaminations of ecosystems. In addition, this study's results demonstrated the effectiveness of GLMMs as a powerful and rigorous statistical analysis tool and the need for new statistical methods.

6.2. Further works

In my research, one of the important points is to improve statistical analysis. As shown in chapter 5, I have been very successful to apply a generalized linear mixed-effects model (GLMM) to ecotoxicological data and this is one of the first ecotoxicological reports to demonstrates the effectiveness of GLMM to stress the importance of each factor influencing the results. Besides, GLMM is very potential to alter the conventional analysis, namely ANOVA. However, I also declare the unsolvable problem of *p* value associated with GLMM for multiple comparisons. Multiple comparisons are known as post hoc tests for categorical variables, are the inevitable practice in ecotoxicological studies. I give the gentle example of disadvantage for *p* value working with categorical variables as follows:

To control type I error at a level of 5% for 21 simultaneous hypothesis tests, p adjustment can be achieved by, for instance, the Bonferroni method.

Bonferroni method, adjusted $\alpha = \frac{\sum_{i=1}^{21}(a_i)}{n_{\alpha_i}} = \frac{0.05}{21} = 0.002$

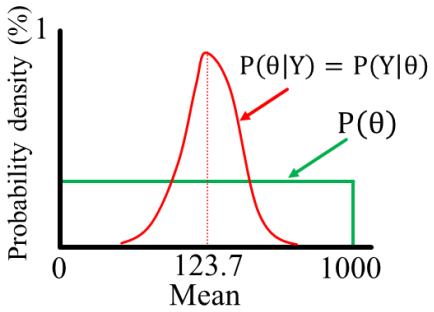
If *p* value in each pair < 0.002 (not 0.05), we say “significant”. Therefore, this fact raises the problem of power loss to reject the null hypothesis when categorical variables contain many levels.

Accordingly, in chapter 5, GLMM also faces this problem and no solution was given. As the further work, I suggest the potential remedy to compromise the problem of *p* value associated with categorical variables, namely GLMM in Bayesian frameworks. The basis for this suggestion is that Bayesian analysis does not need to use *p* value, it is a more natural way for the hypothesis test. I also present a simple example to imagine how Bayesian analysis works.

Bayes's theorem: $P(\theta|Y)=\frac{P(Y|\theta)P(\theta)}{P(Y)} \propto P(Y|\theta)P(\theta)$

- $P(\theta|Y)$ is posterior distribution or result
- $P(Y|\theta)$ is likelihood
- $P(\theta)$ is prior distribution or prior belief

BOD: 150.5; 130.2; 90.4, we would like to estimate the mean value:



Y ~ Normal(Mean, Variance)

Mean ~ Uniform (0, 1000)

Here, the important thing is the prior distribution which means the knowledge about the results. Whatever the prior distribution is (we know or do not know it), I eventually can get the posterior distribution of the result. As we see, our result is the probability distribution of the mean but not the highest probability value in conventional analysis based only on likelihood.

Therefore, given one more dataset of BOD and the task is that we will compare to means. The simple way flowing Bayesian framework is that the find two posterior distribution of two means and the comparison between them is directly done. A reminder that, in the conventional method, to compare to means, we should do:

$$t = \frac{\text{Mean}_1 - \text{Mean}_2}{\sqrt{\frac{\text{SD}_1}{\text{n}_1} + \frac{\text{SD}_2}{\text{n}_2}}}$$

And therefore, *p* value was generated. If increasing groups to compare, we will again face the problem of simultaneous hypothesis tests as above mentioned.

Herein, we have demonstrated how Bayesian analysis works to compromise the problem of *p* value associated with categorical variables. This is my idea for further work.

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APPENDIX

REVIEWS OF ANTIBIOTICS AND CYANOTOXINS

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Fig. A-6. The proportion of total healthy neonates produced by *D. magna* nourished in 5000 μg L^{-1} antibiotic exposure in relation to total healthy neonates produced by *D. magna* nourished in the control considering the proportion of flasks with offspring degradations. Data points represent each birth (e.g., birth 1: b1).

Table A-1. Occurrences of investigated antibiotic residues in various environmental matrices in the period of 2010–2020 (μg L^{-1}).

	CFX	**OFX**	**GFX**	**TC**
PWWTP	4800; 4750; 4650; 3870; 3750; 21.6; 7.84; 6.2; 2.95; 1.25; 0.938; 0.091; 0.031; 0.002	3330; 3200; 3200; 2680; 2660; 23.8; 15.71; 7.5; 7.3; 1.03; 0.35; 0.12; 0.09; 0.0064		2600
HWWTP	53.3; 40.2; 18; 7.63; 3.76; 3.08; 0.78; 1.4; 1.28; 0.5; 0.35	66; 19.8; 15; 7.4; 4.39; 2; 0.24		0.15
MWWTP	5015.6; 17.7; 14.11; 3.4; 2.29; 2.05; 0.66; 0.48; 0.478; 0.45; 0.354; 0.313; 0.304; 0.28; 0.179; 0.1748; 0.148; 0.135; 0.102; 0.091; 0.075; 0.07; 0.055; 0.052; 0.022; 0.019; 0.014; 0.012; 0.0061	542.5; 8.637; 2.33; 2.2; 0.925; 0.86; 0.548; 0.54; 0.434; 0.38; 0.225; 0.22; 0.195; 0.195; 0.191; 0.1718; 0.15; 0.13; 0.117; 0.1; 0.08184; 0.074; 0.059; 0.023; 0.023; 0.013; 0.012; 0.0055; 0.0043	3.7; 0.056	0.63; 0.231; 0.009
LAE	2.1; 0.2; 0.142; 0.029	0.159; 0.048; 0.035		9
River	5528.9; 41.84; 15; 14.3; 13.6; 8.87; 6.67; 3.58; 3.035; 1.729; 1.537; 1.523; 1.44; 1.3; 1.3; 1.168; 1.09; 0.74; 0.62; 0.557; 0.5514; 0.509; 0.346; 0.322; 0.288; 0.277; 0.262; 0.26; 0.234 (truncated)	318.1; 11.7; 8.77; 2.93; 2.89; 2.87; 1.9; 1.73; 0.647; 0.58; 0.57; 0.533; 0.53; 0.4; 0.39; 0.38; 0.34; 0.33; 0.32; 0.22; 0.21; 0.182; 0.18; 0.16; 0.14;	0.48; 0.042	1.9; 0.0087

		0.138; 0.12; 0.12 (truncated)
Lake	27.31; 18.39; 10.72; 0.822; 0.123; 0.115; 0.115; 0.112; 0.096; 0.063; 0.06; 0.044; 0.036; 0.035; 0.029; 0.023; 0.019; 0.018; 0.014; 0.0058; 0.0048; 0.0027	4.53; 3.72; 3.33; 0.518; 0.208; 0.183; 0.183; 0.135; 0.086; 0.083; 0.033; 0.032; 0.021; 0.0094; 0.009; 0.009; 0.00894; 0.005; 0.0023; 0.001; 0.00053

Collected data is maximum values at each sampling location (e.g. each WWTPs or each river), otherwise the average values.

Table A-2. Molar concentrations (μM) of single antibiotic (CFX–ciprofloxacin, DFX–delafloxacin, GFX–gatifloxacin, OFX–ofloxacin, and TET–tetracycline) and fractions of each antibiotic in the antibiotic cocktail (MIX) in ecotoxicological tests.

		Nominal concentrations in weight by volume (mg L^{-1})								Fractions in MIX (%)
	Antibiotic	0	0.625	1.25	2.5	5	10	20	40	
Nominal concentrations in molarity (μM)	CFX	0	1.89 (0.38)	3.77 (0.75)	7.55 (1.51)	15.09 (3.02)	30.18 (6.04)	60.37 (12.07)	120.74 (24.15)	23.28
	OFX	0	1.73 (0.35)	3.46 (0.69)	6.92 (1.38)	13.84 (2.77)	27.67 (5.53)	55.34 (11.07)	110.68 (22.14)	21.34
	GFX	0	1.66 (0.33)	3.33 (0.67)	6.66 (1.33)	13.32 (2.66)	26.64 (5.33)	53.28 (10.66)	106.55 (21.31)	20.54
	DFX	0	1.42 (0.33)	2.84 (0.57)	5.67 (1.13)	11.34 (2.27)	22.69 (4.54)	45.37 (9.07)	90.74 (18.15)	17.49
	TET	0	1.41 (0.28)	2.81 (0.56)	5.63 (1.13)	11.25 (2.25)	22.50 (4.50)	45.00 (9.00)	90.01 (18.15)	17.35

Values in brackets indicate molar concentrations (μM) of single antibiotics in the antibiotic cocktail with respect to the tested concentration.

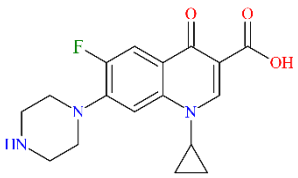
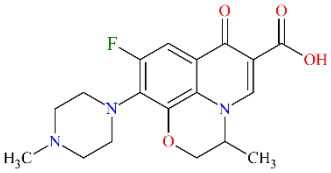
Table A-3. Statistical summary of the effects of birth sequence and exposure conditions on the thoracic limb rate in five single antibiotic tests (CFX–ciprofloxacin, DFX–delafloxacin, GFX–gatifloxacin, OFX–ofloxacin, and TET–tetracycline) and an antibiotic cocktail test (MIX–mixture of five antibiotics).

Test	*df.*	Statistic	*p*-value
Between controls (5 controls) corresponding to births 2–6			
One-way ANOVA	4, 55	$F = 21.61$	< 0.001
Residual normality–Shapiro-Wilk test	60	$W = 0.964$	0.074
Homogeneity of variance–Levene's test	4, 55	$F = 1.675$	0.169
DFX			
One-way ANOVA	7, 72	$F = 1.389$	0.223
Residual normality–Shapiro-Wilk test	80	$W = 0.987$	0.628
Homogeneity of variance–Levene's test	7, 72	$F = 1.472$	0.191
GFX			
One-way ANOVA	5, 54	$F = 20.45$	< 0.001
Residual normality–Shapiro-Wilk test	60	$W = 0.975$	0.245
Homogeneity of variance–Levene's test	5, 54	$F = 1.635$	0.167
TET			

One-way ANOVA	7, 72	$F = 1.033$	0.416
Residual normality–Shapiro-Wilk test	80	$W = 0.980$	0.233
Homogeneity of variance–Levene's test	7, 72	$F = 1.363$	0.234
OFX			
One-way ANOVA	7, 82	$F = 6.442$	< 0.001
Residual normality–Shapiro-Wilk test	90	$W = 0.974$	0.069
Homogeneity of variance–Levene's test	7, 82	$F = 2.626$	0.017
Welch's ANOVA	7, 32.63	$F = 4.768$	< 0.001
MIX			
One-way ANOVA	7, 82	$F = 4.314$	< 0.001
Residual normality–Shapiro-Wilk test	90	$W = 0.976$	0.100
Homogeneity of variance–Levene's test	7, 82	$F = 3.618$	0.002
Welch's ANOVA	7, 31.69	$F = 14.073$	< 0.001
CFX			
One-way ANOVA	7, 72	$F = 11.78$	< 0.001
Residual normality–Shapiro-Wilk test	80	$W = 0.954$	0.006

Homogeneity of variance–Levene's test	7, 72	$F = 1.298$	0.264
Kruskal-Wallis H	7	$H = 45.213$	< 0.001

Table A-4. Chemical properties and the current antibiotic occurrences with high concentrations in various aquatic environments.

Chemical properties		
	CFX	**OFX**
Molecular formula	$C_{17}H_{18}FN_3O_3$	$C_{18}H_{20}FN_3O_4$
Molar mass (g mol^{-1})	331.34	361.37
Chemical structure		

Current occurrences of antibiotics in aquatic environments (μg L^{-1})			
	CFX	**OFX**	**References**
Hospital effluents	18^{A}; 53.3^{L}	66^{A}; 19.8^{L}	Ashfaq et al. (2016) Lien et al. (2016)
Pharmaceutical effluents	4800^{H}; 21.6^{T}	3330^{H}; 23.8^{T}	Hussain et al. (2016), Thai et al. (2018)
Municipal WWTP effluents	5015.6^{G}	542.5^{G}	Gothwal and Shashidhar (2017)
Lake	27.3^{G}	4.5^{G}	Gothwal and Shashidhar (2017)
River	41.8^{A}; 5528.9^{G}	318.1^{G}; 11.7^{J}	Archana et al. (2016), Gothwal and Shashidhar (2017), Jiang et al. (2014)

Uppercases indicate the first letters of authors according to the reference column

Table A-5. Analytical results for ciprofloxacin (CFX) and ofloxacin (OFX) in the initial and terminal medium of the preliminary test.

Antibiotic	Nominal concentration ($\mu g\ L^{-1}$)	Initial concentration ($\mu g\ L^{-1}$) (n = 3)	Terminal concentration (2 days later) ($\mu g\ L^{-1}$) (n = 3)
CFX	5000	5070 ± 55.7	5033.3 ± 61.1
OFX	5000	5036.7 ± 64.3	5003.3 ± 102.6

Data expressed as mean ± standard deviation.

Fluoroquinolone quantification by High-Performance Liquid Chromatography equipped with Photodiode Array Detection (HPLC-PDA).

The method of fluoroquinolone measurements including CFX and OFX was found in the supplementary material of a previously published article (Dang et al., 2021).

Table A-6. Model selection results using the generalized linear mixed-effects models (GLMMs) based on Poisson distribution for brood size.

	Model	cAIC	Deviance	logLik
1	Brood size ~ Treatment + Birth + Time + Treatment × Birth + Treatment × Time + Birth × Time + Treatment × Birth × Time + (1|replica)	924.3	3561.7	−1780.9
2	Brood size ~ Treatment + Birth + Time + Treatment × Birth + Treatment × Time + Birth × Time + (1|replica)	1066.5	3746.4	−1873.2
3	Brood size ~ Treatment + Birth + Time + Treatment × Birth + Treatment × Time + (1|replica)	1202.3	3886.8	−1943.4
4	Brood size ~ Treatment + Birth + Time + Treatment × Birth + Birth × Time + (1|replica)	1066.2	3752.8	−1876.4
5	Brood size ~ Treatment + Birth + Time + Treatment × Time + Birth × Time + (1|replica)	1320.9	4016.4	−2008.2
6	Brood size ~ Treatment + Birth + Time + Treatment × Birth + (1|replica)	1202.4	3891.7	−1945.9
7	Brood size ~ Treatment + Birth + Time + Treatment × Time + (1|replica)	1458.4	4157.9	−2079.0
8	Brood size ~ Treatment + Birth + Time + Birth × Time + (1|replica)	1350.3	4051.9	−2025.9
9	Brood size ~ Treatment + Birth + Time + (1|replica)	1502.6	4205.9	−2103.0
10	Brood size ~ Treatment + Birth + Treatment × Birth + (1|replica)	1210.5	3916.0	−1958.0

11	Brood size ~ Treatment + Time + Treatment × Time + (1|replica)	1843.0	4553.0	−2276.5
12	Brood size ~ Treatment + Birth + (1|replica)	1541.5	4260.4	−2130.2
13	Brood size ~ Treatment + Time + (1|replica)	1896.9	4608.8	−2304.4
14	Brood size ~ Treatment + (1|replica)	2033.7	4748.3	−2374.1

Response variable is Brood size

Explanatory variables are Treatment, Birth, and Time

Random variable is replica

The smaller cAIC indicates the better model

Table A-7. Model selection results based on cAIC at each birth in the birth sequence using GLMMs.

	Brood size ~ Treatment × Time + (1|replica)	**Brood size ~ Treatment + Time + (1|replica)**	**Brood size ~ Treatment + (1|replica)**
Birth 1	72.56687	54.01394	53.44496
Birth 2	60.94766	47.49653	41.90557
Birth 3	80.468	60.92868	53.24695
Birth 4	108.2828	92.60218	67.37465
Birth 5	107.6317	88.86293	87.26056
Birth 6	108.8245	84.33784	74.51475
Birth 7	75.9488	68.6743	68.50013
Birth 8	71.73612	68.41958	55.49211
Birth 9	58.84992	78.60827	73.5108
Birth 10	NaN*	19.49061	19.19232

Response variable is Brood size

Explanatory variables are Treatment and Time

Random variable is replica

The smaller cAIC indicates the better model

* indicates the random effect on the brood size is too small compared to the main effects

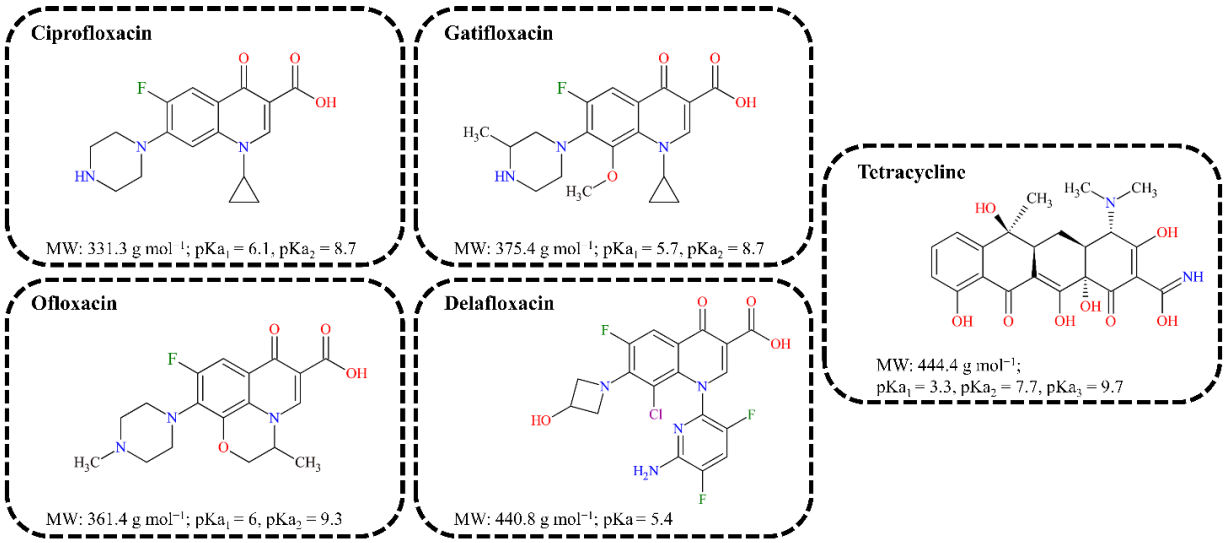


Fig. A-1. Molecular structures, molecular weights, and physical properties of five tested antibiotics.

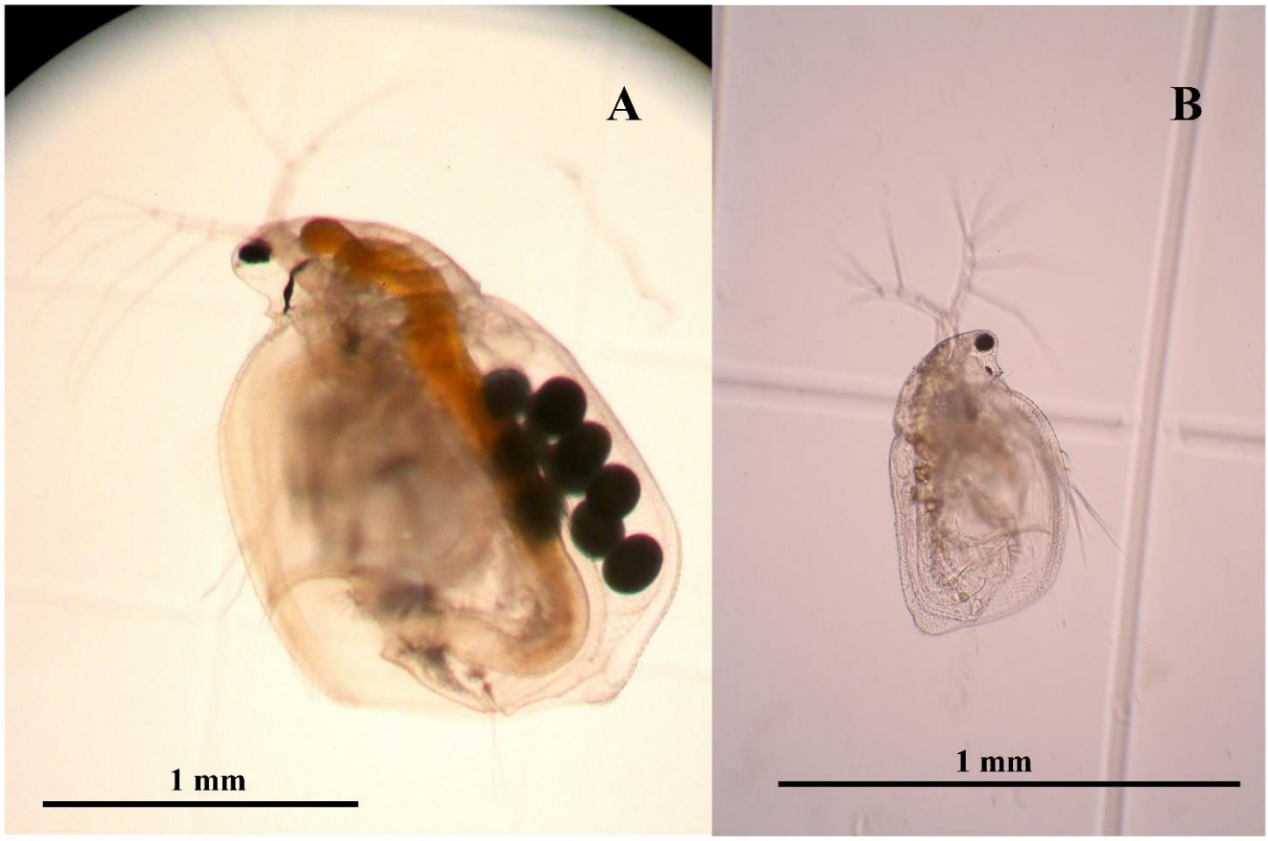


Fig. A-2. 40-day old *Simocephalus vetulus* (A) and 24-hour old *Simocephalus vetulus* (B) nourished in laboratory conditions.

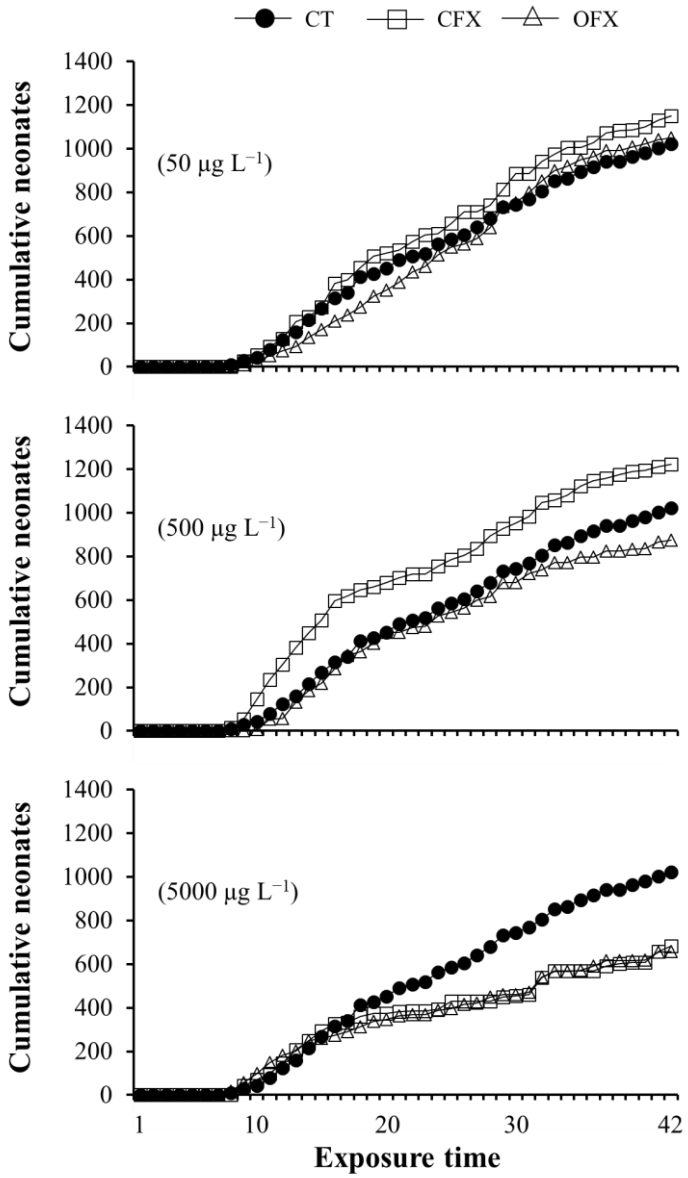


Fig. A-3. Cumulative neonates of each exposure and the control for 42 days.

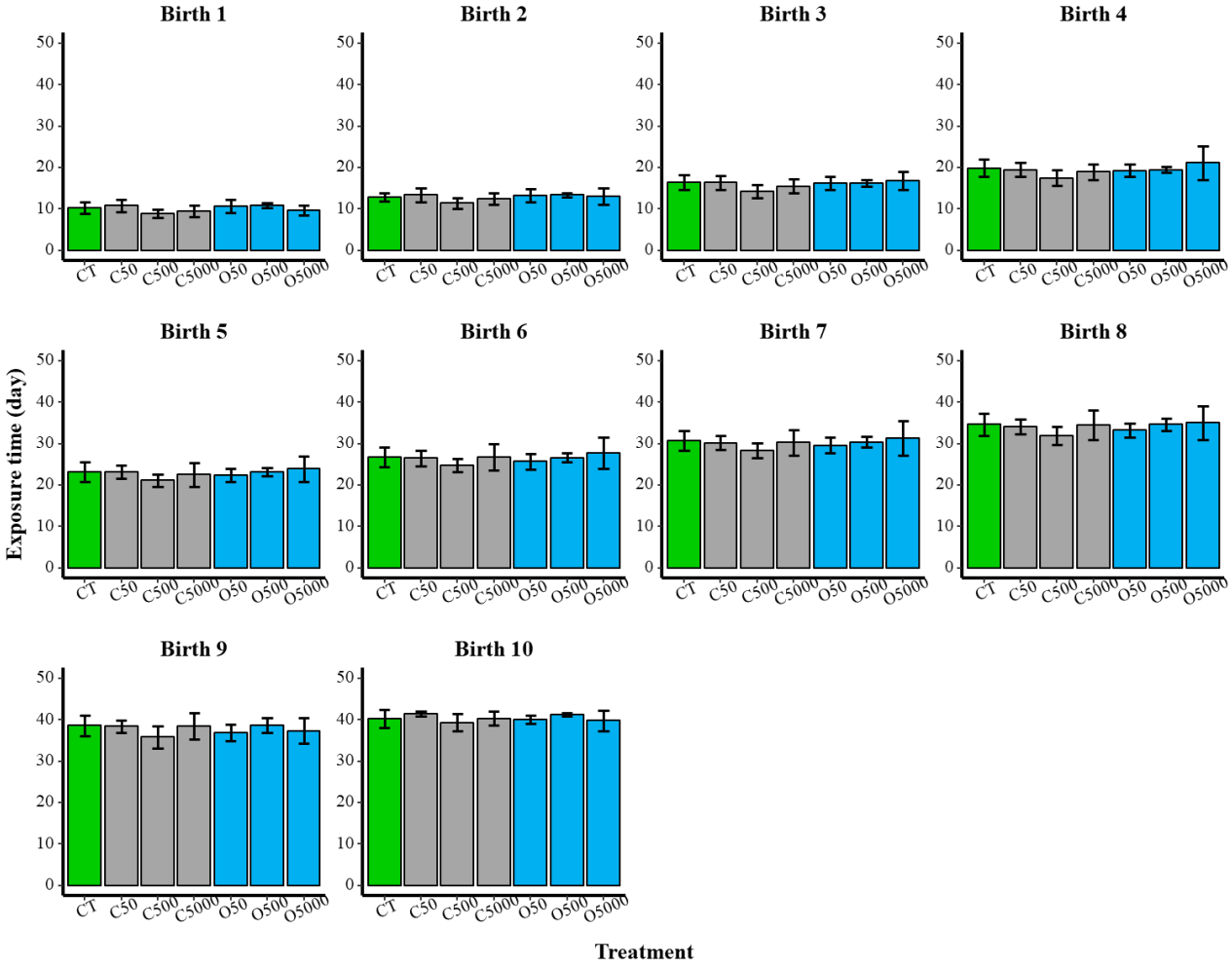


Fig. A-4. The exposure time (days from the start of exposure test) for the exposure conditions at each birth in the birth sequence. Data are expressed as mean ± standard deviation.

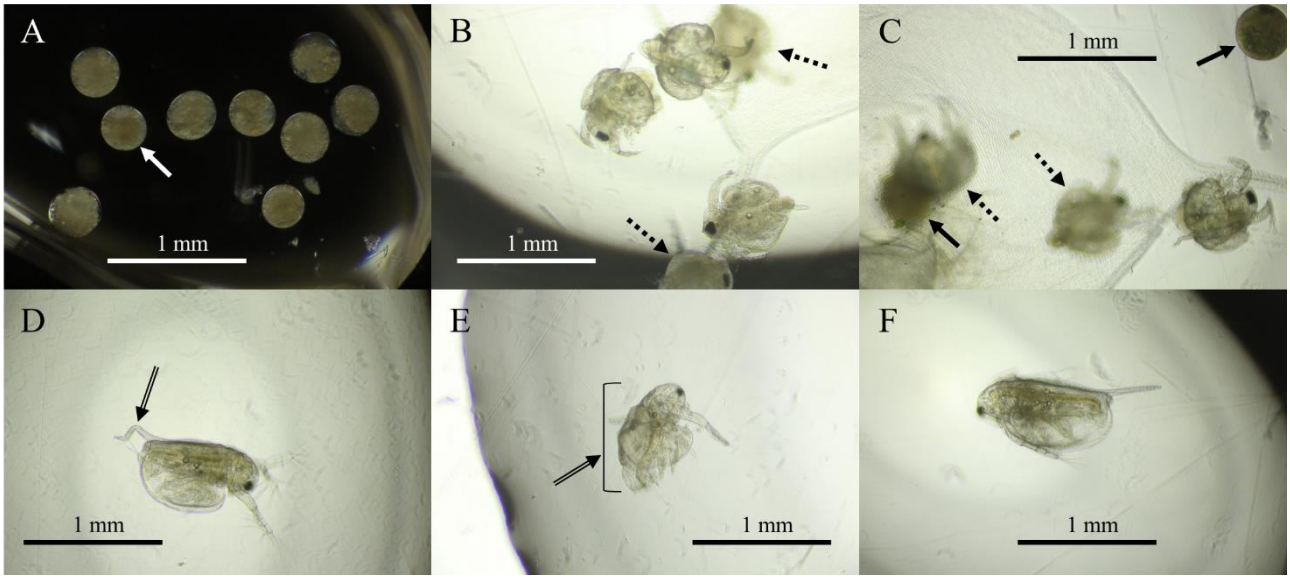


Fig. A-5. The images demonstrated the brood chambers containing dead eggs (A, solid arrow), dead neonates (B, dashed arrow), dead eggs together with dead neonates (C), a malformed neonate with a broken tail spine (D, hollow arrow), a malformed neonate with an abnormal body shape (E, hollow arrow), and a control neonate with a normal tail spine and body shape (F).

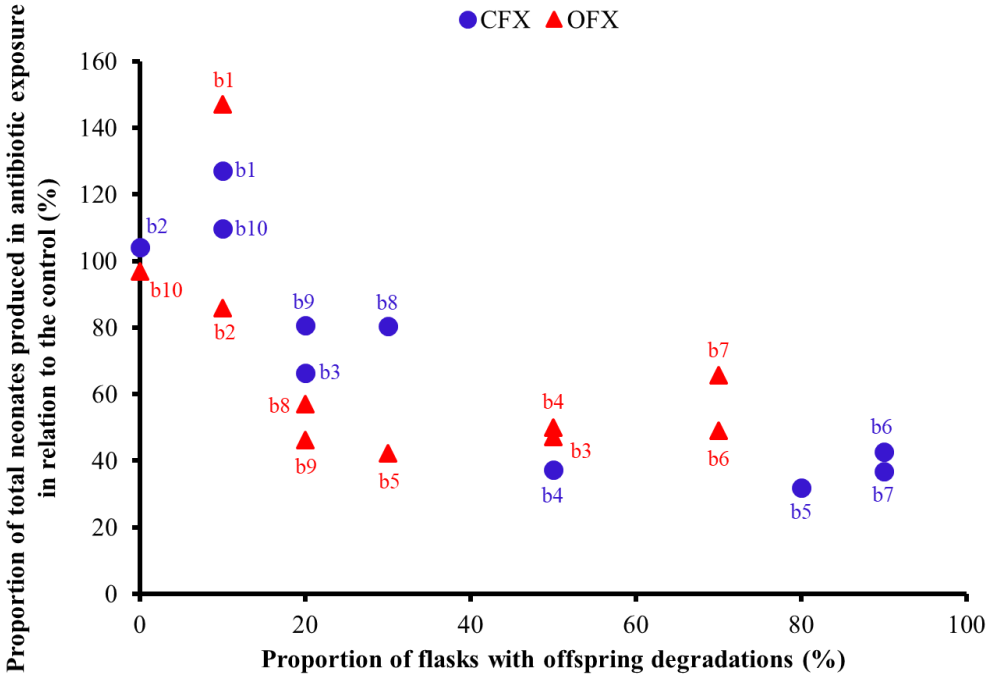


Fig. A-6. The proportion of total healthy neonates produced by *D. magna* nourished in 5000 µg L^{-1} antibiotic exposure in relation to total healthy neonates produced by *D. magna* nourished in the control considering the proportion of flasks with offspring degradations. Data points represent each birth (e.g., birth 1: b1).